Inhibition of the Lethality of Bleomycin A₅ in L-Cells by Hirudonine

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SUMMARY

The lethality of several individual bleomycin derivatives, i.e., the spermidine derivative \(A_5\), the dimethyl sulfonium aminopropyl derivative \(A_2\), and the agmatine derivative \(B_2\), was compared on mouse fibroblasts. The spermidine derivative, bleomycin \(A_5\), was the most toxic, producing more than a 2-log drop in clonability in 50 hr at 20 μg/ml. 6-Azauridine, a relatively nonlethal inhibitor of RNA synthesis and cell multiplication, produced a 60% decrease of adenine incorporation into nucleic acids without inhibiting the lethal action of \(A_5\). This result differed from the effects of inhibition of RNA synthesis on the lethality of \(A_5\) in Escherichia coli. Hirudonine (1,8-diaminopropamide) markedly and specifically inhibited the lethal effects of \(A_5\) in L-cells but not in \(E. coli\). However, hirudonine did not affect the toxicity of \(A_2\) and \(B_2\), separately or together, as it did in the mixture used clinically. Nor did arcaine (diamidinoputrescine) reduce the lethality of the agmatine (monoaaminoputrescine) derivative, \(B_2\).

INTRODUCTION

Natural and synthetic active bleomycins are comprised of a single glycopeptide that is terminated by an amide of 1 of many different polycationic bases (11). The bases of the natural bleomycins appear to be derived in most instances from one or another branch of polyamine metabolism. These antibiotics, which are active antitumor agents, are limited in their effectiveness by their toxicity to lung tissue, as summarized in Ref. 4. We have asked whether the natural polyamines might alleviate this limited toxicity and have begun to study the requirements for cellular lethality in 2 model systems. We have reported (2) on the extraordinary toxicity of \(A_5\) to \(E. coli\) compared to the toxicity of \(A_2\) and \(B_2\), which are the major components of the clinically utilized mixture of bleomycins. These toxicities are manifested in cells by loss of the ability to multiply despite cell enlargement and continuing synthesis of DNA, RNA, and protein (2). Although we could not detect a significant protection by spermidine against \(A_5\) toxicity, the lethality was specifically prevented by blocking RNA synthesis (2). Therefore, we undertook to see whether a similar phenomenon could be detected with mouse fibroblasts (L-cells) in culture.

Although \(A_5\) is significantly more toxic to these cells than is \(A_2\) or \(B_2\), the differences among effects of bleomycins on L-cells are less striking than in \(E. coli\) cultures. It is not possible to block RNA synthesis in L-cells in a nonlethal and complete manner, but an extensive, relatively nonlethal inhibition of RNA synthesis was effected by 6-azauridine, which inhibits the orotate pathway (5). However, such cells were not protected against \(A_5\). Nevertheless, a partially effective specific inhibitor against this lethality of \(A_5\) was found in the diamidinospermidine, hirudonine (1, 9). Another diamino derivative of putrescine, arcaine (1, 9), did not protect as well against \(A_5\) or a more likely candidate, \(B_2\). The structures of these amines and guanido derivatives are presented below:

![Chemical structures of amines and guanido derivatives](image)

MATERIALS AND METHODS

Cells. The growth and multiplication of mouse fibroblasts (L-cells) in suspension culture and the estimation of cell number and size in the Coulter counter have been described (3). The plating technique for determination of cell

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2 Miss L. Lap, Commander, U. S. Naval Reserve Nursing Corps, died on July 16, 1976.
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4 The abbreviations used are: \(A_5\), spermidine derivative of bleomycin; \(A_2\), dimethyl sulfonium aminopropyl derivative of bleomycin; \(B_2\), agmatine derivative of bleomycin; \(A_2\) b, trimethylene diamine derivative of bleomycin.
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viability has also been reported (8). Cultures of cells in the exponential phase were diluted in fresh complete media (7) to 4 to 5 × 10^8 cells/ml and permitted to double to 10^9 by growth for 20 hr. The test compounds were then added to the exponential cultures. Studies on the incorporation of isotopic compounds into nucleic acids were carried out as described earlier (7).

**Chemicals.** Various species of copper-free bleomycins were obtained from Bristol Laboratories, Syracuse, N. Y., with the assistance of Dr. J. Douros of the National Cancer Institute. These substances, for which toxicities to E. coli have been reported (2), were bleomycinic acid, A5, A2-b, B2, and A2. A stock solution of 1 mg/ml in H2O was stored at −20°. 6-Azauridine and 2'-deoxycytidine were obtained from P-L Biochemicals, Milwaukee, Wis. 3-Deazacytidine was obtained from ICN Nucleic Acid Research Institute, Irvine, Calif. Hirudonine sulfate and arcaine sulfate were synthesized by the method of Robin and van Thoai (9); the elementary analyses (carbon, hydrogen, nitrogen) of the isolated substances were those of the pure salts.

**RESULTS**

**Lethality of A5.** Chart 1 presents a comparison of the lethality to L-cells of 2 concentrations of A5. It can be seen that 20 μg/ml were required to produce an exponential decrease to a survival of less than 1% in viable cells in a 48-hr period. In a comparable period of about 2 generation times, 1 μg A5 per ml produced a greater than 10^-3 drop in survival in E. coli (2). Chart 1 (inset) shows that, at 31 hr after addition of A5, the cells were greatly enlarged, particularly in the culture containing 20 μg/ml. At 10 μg/ml there was virtually no lethality for 24 hr. At 31 hr most of the cells exposed to this concentration of drug were enlarged but to a lesser extent than at 20 μg/ml.

**Lethality of Various Bleomycins.** Chart 2 presents a comparison of the lethality to L-cells of various bleomycins at 20 μg/ml. It can be seen that at this concentration bleomycinic acid was only slightly inhibitory, whereas A2-b was approximately as inhibitory as was A5. In tests on E. coli, A2-b was far less toxic than was A5 (2).

A5 and B2 were consistently less lethal than was A5; the former antibiotics produced perhaps one-tenth as much cell kill as did A5. These differences among effects of bleomycins on L-cells, although significant, are far less so than in E. coli (2). B2 was consistently more toxic in this system than was A5; in the mixture used clinically, A5 was frequently present in amounts 1.5 to 2 times that of B2.

In Chart 2 (inset) it can be seen that B2 and A5 affected cell size similarly, whereas A5 was intermediate in its effect. Bleomycinic acid permitted an exponential increase in cell number for at least a full day. A5 frequently permitted such an increase for 18 hr, followed by an abrupt arrest in multiplication and cell death. In contrast to those effects, A5, A5-b, and B2 produced an immediate and very considerable inhibition of the increase in cell number. The intermediate sizes of cells treated with A5 for 31 hr seemed to reflect a later inhibition of division by this drug.

**Attempted Prevention of Bleomycin Toxicity by Polyamines.** In studies with A5-b at 20 μg/ml, which caused a 2-log drop in viability in 48 hr, neither 10^-2 M putrescine nor 10^-2 M spermidine decreased the cell kill significantly in this time.

In studies with A5 at 20 μg/ml, which also produced a 2-log drop in 48 hr, 10^-2 M spermidine added to A5 caused a lag of 24 hr in cell kill, followed by a precipitous killing to the level of A5 alone. A similar result had been seen in a temporary protection by high concentrations of spermidine against A5 with E. coli (2). In studies with the L-cell system, the size distributions of cells treated with A5 and cells treated simultaneously with A5 plus spermidine were similarly heterodisperse at 24 and 48 hr. A brief protection against A5 by 10^-2 M spermidine (data not shown) had not prevented cell enlargement, i.e., synthesis and cell growth.

**Inhibition of Nucleic Acid Synthesis and A5 Lethality.** In our studies on E. coli, we had observed that the lethality of A5 was expressed without significantly affecting the rates of synthesis of DNA, RNA, and protein (2). At 20 μg/ml, A5 has been reported to reduce DNA synthesis in mouse fibroblasts about 75% in 2 hr and to inhibit RNA and protein synthesis.
about 10 and 20%, respectively (12). In our studies on the effect of A₅ (20 μg/ml) on [³H]thymidine and [¹⁴C]uridine incorporation on L-cells (3.7 x 10⁶ cells/ml), no differences were found in these incorporations over a 4-hr period in control and treated cultures. After 24 hr, the A₅-treated culture was found to incorporate thymidine at a rate similar to the initial culture, unlike the control in which the rate of thymidine incorporation had doubled. After 24 hr, however, the A₅-treated culture had almost doubled its rate of uridine incorporation.

The lethal effects of A₅ and B₂ (J. I and S. S. Cohen, unpublished data) required RNA synthesis in E. coli (2). We have attempted to see whether this surprising result was also obtained with L-cells. Despite the large amount of work on the inhibition of RNA synthesis in mammalian cells, few attempts have been made to determine whether cells inhibited in RNA synthesis were also killed. For example, actinomycin D is an excellent inhibitor of RNA synthesis, but is also lethal under the conditions of prolonged incubation necessary to explore the lethality of bleomycin. Among the few compounds that appeared to be capable of inhibition of RNA synthesis without extensive killing were 3-deazauridine (10), 3-deazacytidine (6), and 6-azauridine (5); the effects of these agents on RNA synthesis are reversed by natural ribosyl nucleosides, such as uridine and cytidine. This reversal is particularly effective in the presence of deoxycytidine, which appears to prevent the lethal effects accompanying an inhibition of DNA synthesis.

At 100 μg of 3-deazacytidine per ml, cell number increased 20% in 24 hr and fell to 80% of the original cell number at 48 hr. The addition of 100 μg of deoxycytidine per ml to 3-deazacytidine at this concentration permitted cell multiplication at one-half the rate of the control. In any case neither of these inhibitor combinations inhibited the course of A₅ killing over a 33-hr interval.

This result led us to test 6-azauridine with or without deoxycytidine as an inhibitor of nucleic acid synthesis. It was found that, after a few hr of treatment, 10 μg of 6-azauridine per ml totally inhibited the increase of cell number for 24 hr without killing. The cells began to die slowly 32 hr after addition of this agent. In the presence of 6-azauridine plus 40 μg of deoxycytidine per ml, the total number of cells as well as the number of viable cells increased slowly. Nevertheless, as in the experiments with 3-deazauridine, neither treatment markedly affected the lethality of A₅.

In short-term studies on uptake of [⁸-¹⁴C]adenine into the nucleic acids of L-cells, it was found that 6-azauridine produced its maximal inhibition (60%) after 3 hr. On the other hand, in the presence of deoxycytidine, the uptake of adenine into nucleic acids was normal for at least 7 hr.
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Accordingly, these experiments on bleomycin killing in the presence of 6-azauridine were repeated with the addition of A5 5 hr after inhibition with 6-azauridine. The addition of 6-azauridine to A5 permitted the same rate of cell kill whether added before the antibiotic or at the same time. Preincubation with deoxycytidine and 6-azauridine did not significantly affect the rate of cell kill after addition of A5. These results indicate that, contrary to results with E. coli, the extensive inhibition of RNA synthesis did not hinder the lethal action of A5.

Prevention of the Lethality of A5 by Hirudonine. It was found that 10^-4 M hirudonine was partially effective in blocking A5 at 20 μg/ml. This was in marked contrast to the ineffectiveness of 10^-2 M spermidine in blocking the toxicity of A5. It can be seen in Chart 3 that 10^-4 M hirudonine was non-toxic, whereas 10^-3 M hirudonine slowed growth rate somewhat. In the presence of A5, 10^-4 M hirudonine protected the cells considerably, whereas at 10^-3 M the compound was reproducibly somewhat less protective. As presented in Chart 3B, hirudonine enabled cultures containing A5 to increase in cell number at a significant rate.

These effects were also obtained in the distribution of cell sizes. Hirudonine (10^-4 M) alone affected cell size somewhat at 24 hr after addition of the compound, whereas A5 alone changed the size distribution greatly. With 10^-4 M hirudonine, a large number of cells remained in the normal size range in the presence of A5.

Specificity of Hirudonine Protection. Aliquots of the same exponentially growing cultures were challenged by 3 different bleomycins, A5, A2, and B2, at 20 μg/ml in the presence or absence of 10^-4 M hirudonine or arcaine. It can be seen in Chart 4 that hirudonine protected against A5 but not against A2 or B2. Arcaine (10^-4 M) protected somewhat against A5 (Chart 4A) but not nearly as well as hirudonine. However, arcaine was ineffective against A2 (Chart 4B) or B2 (Chart 4C).

DISCUSSION

As seen earlier in studies with E. coli, A5 is reproducibly more lethal than the components A2 and B2 of the clinical bleomycin mixture. The latter are known to produce significant lung toxicities, which limit their usefulness in the clinic; at present no way of preventing their long-term toxicity is known. The availability of a partially effective specific protective agent against A5, i.e., diamidinospermidine or hirudonine, offers an additional reason for a serious test of the efficacy of A5 in an animal tumor system. It is obvious that a thorough appreciation of the antitumor activity of a bleomycin will require study with a pure component. For this reason it will be as easy to study A5 as A2 or B2, and it would make sense to test A5, which is more lethal than A2 or B2, and for which significant protection of surviving cells may be obtained with a relatively nontoxic antagonist such as hirudonine.
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