Hyperthermia and Bleomycin Schedules on V79 Chinese Hamster Cell Cytotoxicity in Vitro

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ABSTRACT

The effect of sequence and timing of hyperthermia (43°) and bleomycin on Chinese hamster cells (V79) has been investigated. Hyperthermia preceding bleomycin treatment produced a greater cytotoxic effect than bleomycin treatment preceding hyperthermia. Furthermore, it appears that the combination of hyperthermia and bleomycin becomes less effective if the application of bleomycin is delayed. The enhancement of cytotoxicity with hyperthermia first may be related to the effect of heat on the intracellular bleomycin degradation ability and protein synthesis. V79 cells treated with a protein synthesis inhibitor, cycloheximide, before bleomycin (but not in the reversed sequence) also showed a markedly lower level of survival. As for hyperthermia treatment, pretreatment with cycloheximide did not change the uptake of bleomycin. These results suggest that hyperthermia and cycloheximide have increased the effectiveness of bleomycin and are consistent with the observation on chromosome damage induced by hyperthermia-bleomycin and cycloheximide-bleomycin treatments reported in the literature.

INTRODUCTION

It has been reported that hyperthermia can greatly enhance the cytotoxic effect of bleomycin, an anticancer antibiotic (4, 10). Bleomycin is widely applied in the treatment of human squamous cell carcinomas, testicular carcinoma, and lymphomas. It is important to recall the fact that enhanced cytotoxicity did not result from any additional amount of bleomycin uptake in heat-treated cells (4). Inhibition of cell recovery by hyperthermia is responsible for the enhancement of bleomycin cytotoxicity (4). Subsequently, several studies in vitro (14, 16, 18, 19, 28) and in vivo (12, 13) all confirm that hyperthermia effectively enhances the bleomycin cytotoxic effect. As for the mechanism, Meyn et al. (14) observed that the rate and amount of repair of DNA strand breaks of bleomycin-treated cells at 43° is less than for cells treated at 37°. Thus, these authors echoed the concept forwarded by Braun and Hahn (4). Others have suggested different possibilities such as either an increased induction of initial damage (28) or a modification of the cell cycle (19).

Since hyperthermic temperatures can produce many different cellular effects (for reviews, see Refs. 5 and 29), it is reasonable to expect that hyperthermia could have potentiated the bleomycin effect by many different mechanisms. Since the enhancement was not the result of an increased bleomycin uptake, it is possible that heat-treated cells may have a reduced bleomycin inactivation ability which, in turn, could contribute to part of the enhancement effect. The following discoveries provide the rationale for our investigation. (a) Most mammalian cells contain 2 bleomycin inactivation components, bleomycin hydrolase and a low-molecular-weight fraction (27, 31). These components have been shown to degrade bleomycin and also have been shown to have greater activities in bleomycin-resistant cells (1, 15). (b) It has been suggested that the characteristic resistant phase of the biphasic bleomycin survival curve is due to a bleomycin induction process (26). Furthermore, the bleomycin-induced resistance decays shortly (about 2 hr) after the removal of bleomycin from the experimental system (25, 26, 30). (c) The principal benefit for combining hyperthermia with bleomycin is the elimination of the bleomycin-resistant portion of the survival curve. This can result from the inability of the heat-treated cells to respond to the bleomycin induction process. (d) In addition to cell inactivation, several reports have demonstrated that hyperthermia inhibits cellular nucleic acids and protein synthesis (6, 11, 17). It is also well known that the stability of many proteins may be drastically affected by elevated temperatures. Thus, it seems possible that heat-treated cells contain lower bleomycin inactivation activity. In the experimental results presented in this report, we determined (a) the bleomycin effect on hyperthermia-(pre- or postbleomycin) treated cells; (b) the effect of elevated temperatures on the bleomycin inactivation activity of rat liver extracts; and (c) the role of protein synthesis in the bleomycin cytotoxic effect.

MATERIALS AND METHODS

Cells. A Chinese hamster cell line, V79, was used in all the experiments. The cells were maintained in Costar plastic bottles and in minimum essential medium, with 5% heat-inactivated fetal calf serum and 5% calf serum. Cultures in log growth were trypsinized, and the cells released from the flasks were quickly suspended in medium containing serum. After one wash, the cells were resuspended with complete medium and diluted to appropriate cell densities. Cell survival curves were plotted to show bleomycin effect.

Treatments. All hyperthermia experiments (including 37°) were carried out in water baths at preset temperatures (±0.1°C). Bleomycin (obtained from Dr. W. T. Bradner, Bristol Laboratories, Syracuse, N. Y., as Bleomycin) and cycloheximide (obtained from Sigma Chemical Co., St. Louis, Mo.) were dissolved in 0.9% NaCl solution and were then added at various concentrations to cell suspensions. At the end of the treatment period, the cells were washed twice with complete medium before being plated on plastic Petri dishes. After 6 to 7 days' incubation at 37° in a 5% CO2/95% air incubator, colonies on the plates were fixed with methanol, stained with Giemsa, and counted.

Liver Extracts. Liver from laboratory rats were excised and homogenized in 0.1 M cold phosphate buffer (pH 7.2). In all extraction procedures, the tissue and buffer were maintained at a ratio of 1 g/2 ml. The crude liver extract was prepared essentially with the method described by Yoshikawa et al. (31). Briefly, the liver homogenates were first centrifuged at low speed (12,000 rpm for 60 min) to remove the debris, and then the supernatants were further centrifuged at 100,000 x g for 60

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min. The supernatants from the second centrifugation were then dialyzed overnight against the same buffer used in the homogenization. The resulting crude enzyme preparations were then tested for activity against bleomycin. The fact that some of the preparations failed to show this activity demonstrates the unstable nature of this enzyme (31). The crude enzyme preparation was stored at −80°C.

**Determination of the Bleomycin Inactivation Activity.** Each crude enzyme preparation was divided into 2 portions; one served as a control for the other, which was treated by elevated temperatures. The effect of heat treatment was determined by 2 methods. (a) The same amounts of bleomycin were added to the crude enzymes (heat-treated and control) and incubated at 37°C for 30 min. After this period, the mixture of bleomycin and crude enzymes was used for V79 cell survival experiments to determine the potency of bleomycin. (b) We have begun to use HPLC analysis to determine the amount of bleomycin after incubation with the crude enzyme. A Beckman Model 330 isocratic system (Beckman Instruments, Inc., Irvine, CA.) with a 25- x 4.6-cm reversed-phase (octadecyl silane ultrasphere) column was used for our experiment. The mobile phase was an equal proportion of methanol (20%), ammonium acetate, and water. The flow rate was 1 ml/min at 3000 psi at 21°C.

**Determination of Protein Synthesis.** Essentially the method described by Fuhr (6) was adapted. Briefly, [14C]leucine (300 mCi/mmol; New England Nuclear, Boston, Mass.) was added to various V79 cell suspensions at a concentration of 1 μCi/4 x 10⁶ cells. After incubation at 37°C for 15 to 120 min, a 1-ml cell suspension from each specimen was first transferred to 5 ml of ice-cold 0.9% NaCl solution and then pelleted by centrifugation. The cell pellet was resuspended in 1 ml of water and 1 ml 20% trichloroacetic acid and heated at 90°C for 20 min. The trichloroacetic acid precipitates were collected on Millipore filters, and the incorporated radioactivity was determined.

**Uptake of 57Co-Bleomycin.** 57Co-Bleomycin (1 mCi 57Co per mg bleomycin) was prepared from 57CoCI2 (New England Nuclear) and bleomycin by the method described by Grove et al. (9). Thin-layer chromatography showed that 98% of the radioactivity was associated with bleomycin. 57Co-bleomycin (1.8 μCi) was added to 2.5 x 10⁶ cells and incubated for an additional 30 min at 37°C. The cells were then washed twice with medium before the radioactivity of the samples was determined and compared.

**RESULTS**

Our basic approach to the interaction of hyperthermia and bleomycin was to examine the cytotoxicity in different sequences of treatment. First, the effect of hyperthermia and bleomycin administered simultaneously on V79 cells was determined. Chart 1 shows that the response of V79 cells at 37°C to bleomycin as a function of exposure time yielded a characteristic biphasic survival curve. If the treatment of bleomycin was carried out at 43°C, the bleomycin-resistant phase changed precipitously. In similar experiments, cell survival as a function of bleomycin concentration instead of exposure time was determined at 37°C and 43°C for 60 min. Again, the survival of 43°C-treated V79 cells changed dramatically in the bleomycin-resistant phase (Chart 1).

In separated experiments, the survival of V79 cells at 43°C for 60 min alone was about 30%. The second series of experiments were carried out to determine whether there is a difference in the survival response with different sequences of hyperthermia and bleomycin. The experimental protocol was a 1-hr hyperthermia treatment of 43°C either given immediately before or immediately after bleomycin (10 to 50 μg/ml) for 30 min at 37°C. Chart 2 demonstrates the effect of hyperthermia given before and after bleomycin treatment, respectively. It is apparent that hyperthermia given before the bleomycin is much more effective in reducing survival than that given after the bleomycin treatment. From these results, we decided to determine the effect of a time gap between hyperthermia and bleomycin on cytotoxicity. Thus, in the third series of experiments, V79 cells first were incubated at 43°C for 1 hr and then either immediately treated with bleomycin (20 μg/ml) for 30 min at 37°C or incubated at 37°C for 30 min to 4 hr before being treated with bleomycin (20 μg/ml; 30 min at 37°C). Chart 3 shows the relative survival of delayed (T₁) and immediate (T₀) bleomycin treatment after hyperthermia. The results as expressed in ratio of T₀/T₁ suggest that delaying bleomycin treatment after hyperthermia reduces the potential effectiveness of the combined cytotoxic effect.

To test whether hyperthermia treatment may induce intracellular bleomycin inactivation, we prepared cell extracts from rat liver that contained bleomycin inactivation activity. Bleomycin (2.5 mg/0.5 ml of 0.9% NaCl solution) was added to 0.5 ml of liver extract which may or may not have been exposed to 41°C or 43°C for 30 min. The bleomycin/liver extract mixtures were incubated at 37°C for 30 min first and then they were added to V79 cell suspensions at a concentration of 50 μg/ml (bleomycin
Chart 3. Effect of an incubation (at 37°) period between hyperthermia (43° for 60 min) and bleomycin (20 μg/ml, at 37° for 30 min) on the relative survival of V79 cells. T₀, bleomycin treatment immediately after the hyperthermia treatment; T₁, bleomycin treatment delayed for 30 min to 4 hr after the hyperthermia treatment.

The cell suspensions were spun after 30 to 90 min of incubation at 37°, and the cell pellets were washed once before they were plated. Chart 4 shows that bleomycin exposed to the 43°-treated liver extract has a similar cytotoxic effect as control bleomycin, whereas bleomycin exposed to the liver extract that had not been treated by elevated temperatures or treated by 41° has a less cytotoxic effect than the control bleomycin. To further assist us in evaluating the effect of hyperthermia, we have performed some preliminary HPLC experiments to determine whether the liver extracts actually reduce the level of bleomycin. Bleomycin (0.5 mg) was added to 1 ml of liver extract (with or without 45°, 30 min pretreatment) and incubated at 37° for 30 min. The mixtures were then diluted, and 40 μl (containing 15 μg of bleomycin) was used in HPLC determination. In the bleomycin control HPLC chromatograms, we observed 2 peaks as reported by others (21, 22) to be bleomycin A₂ and bleomycin B₂. In addition, HPLC chromatogram of purified bleomycin B₂ obtained from Dr. W. T. Bradner coincided with the second peak of the bleomycin (Blenoxane) control. The liver extract that was not treated by hyperthermia greatly reduced the peak height of bleomycin (data not shown). Since heated liver extract showed less effect on bleomycin level, this result suggests possible degradation of the enzyme.

Since the hyperthermia treatment can disrupt the cellular protein synthesis, we decided to study the inhibition of protein synthesis in relationship to bleomycin-treated cell survival characteristics. After 1 hr, treatment at 43° reduces the rate of protein synthesis at 37° to less than 30% of control cells or cells treated with bleomycin alone. Additional bleomycin (25 μg/ml) to the 43°-treated V79 cells did not reduce the protein synthesis rate further. The experiments in which cells were treated with bleomycin first and then hyperthermia after the bleomycin was washed off showed similar results. To assess the effect of reducing protein synthesis on bleomycin cytotoxicity, we tested the effect of cycloheximide. The rate of protein synthesis in cells treated with cycloheximide (25 μg/ml for 60 min at 37°) was reduced to less than 10% of the rate of control cells. If the bleomycin (added at the same time with leucine) was added to cycloheximide-pretreated cells, the rate of protein synthesis was depressed slightly further than cycloheximide alone (data not shown). Chart 5 shows the result of V79 cells first treated with cycloheximide (25 μg/ml for 60 min at 37°) and then with bleomycin (20 μg/ml) for an additional 10 to 80 min at 37°. Although a 25-μg/ml dose of cycloheximide was neither toxic by itself nor did it change the biphasic-shape bleomycin survival curve, it did markedly lower the level of survival of bleomycin-treated cells. Cycloheximide pretreatment also showed a similar effect on V79 cells treated with 10 to 100 μg/ml of bleomycin for 60 min at 37°. However, adding cycloheximide to bleomycin-pretreated V79 cells had little effect (Chart 6).

The uptake of 57Co-bleomycin in cells treated with hyperthermia (43° for 15 to 60 min) and cycloheximide (25 μg/ml for 30 to 120 min) appeared similar to that in control cells (data not shown).
DISCUSSION

Hyperthermia is one of the best-studied methods to increase the bleomycin cytotoxic effect. More specifically, our results as well as those reported by others (4, 10, 16) all indicate that hyperthermia treatment can greatly modify the bleomycin-resistant component. Our observation emphasizes the following. There is sequence difference in applying hyperthermia and bleomycin. When hyperthermia is applied before or simultaneously with bleomycin, a greater cytotoxic effect is produced than when bleomycin is followed by hyperthermia. A similar observation has been reported that 1 hr at 43° and then treatment with bleomycin (50 μg/ml) for 1 hr at 37° produces about 1 log greater cytotoxicity than the reversed sequence of treatment (4). The fact that the effectiveness of hyperthermia in enhancing the subsequent bleomycin effect decays progressively if the hyperthermia-treated cells are incubated at 37° before being treated by bleomycin agrees with reports by other investigators (4, 12). Certainly, the effects of hyperthermia in interfering with cellular repair of potential lethal damage (4) and in reducing the rate of repair of bleomycin-induced DNA strand breaks (14) are important contributing factors for the overall survival. It is also worth noting the fact that there was no significant difference between heat-bleomycin and bleomycin-heat sequences on the final recovery from the potential lethal damage, and yet in heat treatment the first group consistently showed a substantially large initial cytotoxic effect (4). Likewise, in the analysis of chromosome aberration and SCE by hyperthermia and bleomycin treatment, Vig (28) observed that the hyperthermia treatment enhanced chromosome aberration but showed no effect on SCE induced by bleomycin. Since the SCE has been considered a sensitive indicator of repairable DNA damage, the results also indicate that there is an increase of effectiveness in inducing initial damage. Our results reflect 2 possibilities for heat to enhance the bleomycin effect.

It needs to be reemphasized that if the same amount of bleomycin entered the cells with different bleomycin-degrading activities, the cell with lower degrading activity would probably suffer a greater bleomycin toxic effect. As reported by others (4), our 57Co-bleomycin uptake study indicates that hyperthermia treatment does not increase the cells' permeability to bleomycin. Thus, the reduced bleomycin degradation ability observed in hyperthermia-treated liver extracts may be at least partly responsible for the increased initial chromosome aberration (28) as well as enhanced cytotoxicity. It seems that the effect of hyperthermia on the bleomycin degradation ability may also be involved with the apparent difference in the sequence of hyperthermia and bleomycin. In cells treated by hyperthermia first (Chart 2A), the bleomycin inactivation ability is reduced and as a consequence the effectiveness of intracellular bleomycin to initiate damage would be greater than for cells treated by bleomycin first (Chart 2B) or without hyperthermia treatment.

The other related possibility is by suppressing the development of a bleomycin-resistant component. Since the bleomycin-induced resistance decays within 2 to 4 hr (25, 26, 30), any resistance associated with the bleomycin inactivation enzyme probably has a short half-life. If so, the effect of hyperthermia on protein synthesis described in this report and by other investigators (3, 6) should play a role in bleomycin cytotoxicity. The result of the heated liver extract experiments seems to suggest that even if new enzyme synthesis can continue, the new enzyme would be equally susceptible to heat inactivation. The cycloheximide results support this viewpoint. Cycloheximide at a concentration of 25 μg/ml effectively inhibits the cellular protein synthesis but is not cytotoxic for a 2-hr exposure or after the uptake of 57Co-bleomycin. The enhanced cytotoxicity without an obvious increase of intracellular drug concentration is similar to the situation of cells treated by hyperthermia. An interesting finding in this context is that only the cycloheximide treatment before bleomycin can augment the bleomycin cytotoxicity. In comparison with the hyperthermia experiments (Chart 2A), the cycloheximide treatment (before bleomycin) (Chart 6A) may have inhibited the induction of additional resistance, but this treatment should not prevent the bleomycin inactivation enzyme originally present in the cells from contributing to the development of the overall survival characteristics. The results of the cell survival experiments of cycloheximide and bleomycin appear to be consistent with the observation of Sognier et al. (23). These authors showed that in the presence of cycloheximide (25 μg/ml), bleomycin consistently induces a greater number of chromatid breaks in Chinese hamster ovary cells than bleomycin alone.

It should be pointed out that the effect of heat on the bleomycin degradation enzyme is not unique. Several particularly well-known enzymes that may also be involved in hyperthermia enhancement of the bleomycin effect in Chinese hamster cells have been shown to be very heat sensitive. For example, it has been reported that heat can inactivate DNA polymerase α and β (24) and that the indirect heat effect on protein synthesis prevents the synthesis of new ornithine decarboxylase (3, 7, 8). Since these DNA polymerases (particularly the β) are involved in bleomycin-induced unscheduled DNA synthesis (20), heat effect on these enzymes may be responsible for the observation of Meyn et al. (14). Recently, it has been demonstrated that an ornithine decarboxylase inhibitor, DL-α-difluoromethylornithine, can potentiate the bleomycin effect on a parasite, Trypanosoma, and apparently polyamines can cancel this enhancement effect (2).

Finally, our results point out the importance of the sequence and timing of combining bleomycin with other agents. In combination with hyperthermia, bleomycin should be given after or simultaneously with hyperthermia. If the 2 treatments have to be applied separately, bleomycin should follow the hyperthermia as soon as possible. Indeed, the difference in the scheduling of these 2 treatments in vivo on Lewis lung tumor growth delay or
anti-KHT tumors in mice has been observed (12, 13). Especially, the tumor growth delay observed on combined treatment of hyperthermia and bleomycin cannot be observed with a 4- or 24-hr gap in between these 2 treatments (12). However, the hyperthermia used in this type of combination probably should be limited to localized treatment to avoid a possible increase in the bleomycin side effects. Furthermore, in combination chemotherapy involving bleomycin and other agents which are capable of interfering with protein synthesis, the general guide for the hyperthermia-bleomycin combination may also be applicable.

REFERENCES


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