[CANCER RESEARCH 35, 2921 2927, November 1975]

# Enhanced Cell Killing by Bleomycin and 43° Hyperthermia and the Inhibition of Recovery from Potentially Lethal Damage[1]

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## SUMMARY

The effect of hyperthermia on bleomycin (BLEO) toxicity and repair was studied in "unfed" monolayer cultures of Chinese hamster cells. Synergy of toxicities was observed with simultaneous exposure to BLEO and 43°. For example, when cells were exposed for 1 hr to BLEO (40 $\mu$g/ml) at 43°, survival was reduced to $4 \times 10^{-5}$; separately, hyperthermia and 37° BLEO exposure each resulted in a survival of 20%. Heating at 43° prior to drug exposure at 37° also produced substantial sensitization, indicating that the primary sensitizing effect involved cell damage rather than an increased rate of drug action; 41° produced only modest cell sensitization to BLEO and the effect was not retained in cells heated prior to drug exposure.

No increase in [$^{14}$C]BLEO uptake was observed at 43° over that at 37°, and thus the increased cytotoxicity was not correlated with a gross change in cell permeability to BLEO, although increased drug availability to particular sensitive targets could not be ruled out. Studies of the repair kinetics after different 43° BLEO protocols demonstrated that most of the cells sustaining potentially lethal damage rapidly recovered. However, 43° hyperthermia inhibited this recovery and, with increasing durations of 43° exposure, the fixation of potentially lethal damage was enhanced. Because of the substantial repair of BLEO damage observed *in vivo*, the possible usefulness of hyperthermia as an adjunct to BLEO therapy is discussed.

## INTRODUCTION

Recently, a great deal of activity has centered on the potential of hyperthermia (temperatures above 37°) as an adjunct modality in cancer therapy. Increased heat sensitivity of malignant over normal tissue has been observed in several instances (6, 36). *In vitro*, selective heat sensitivity has also been observed (11, 23, 24, 34), and was recently quantified for normal and SV40-transformed human fibroblasts (20). However, this response may not be universal (19, 31).

Cell sensitivity to hyperthermia has been related to the effects of growth phase, nutritive state, and ambient medium in Chinese hamster cells *in vitro* (12). Plateau phase cultures were found to be more heat sensitive than exponential cultures, with the sensitivity increasing in the order: exponential < unfed plateau < fed plateau (in fed plateau cultures, the medium is exchanged daily as cells approach confluence). Recovery from heat damage proceeded rapidly ($T_{1/2}$ ~30 min in full medium) in plateau cultures, but its magnitude in unfed cultures was greatly reduced (G. Hahn, E. Shiu, G. Li, and J. Braun, unpublished data).

Exactly how mild hyperthermia kills cells has not been established with certainty. Protein damage has received the greatest amount of interest, and a significant amount of evidence has mounted in its favor. In Chinese hamster ovary cells, an Arrhenius plot of rate of cell killing *versus* temperature$^{-1}$ yielded a cell inactivation energy of 140,800 cal/mole, a value which corresponds to denaturation energy for several proteins (42). Other workers observed 185,000 cal/mole inactivation energy for Chinese hamster V-79 cells, which was also explained in terms of protein denaturation (18).

Combined toxicity of heat and drugs has been reported for several agents and may involve both an increase of initial damage and an inhibition of repair. In exponential cultures of Chinese hamster V-79 cells, rate constants for tris(aziridinyl)phosphine sulfide toxicity increased regularly for both the drug concentration and temperature of the drug exposure (18), presumably reflecting an increase in the reaction rate for alkylation. Increased drug toxicity at elevated temperatures, due either to additive or synergistic interactions, was also observed for cordecypin, 2-mercapto-1-($\beta$-4-pyridethyl)benzimidazole, L-erythro-$\alpha$,$\beta$-dihydroxybutyraldehyde (10, 32), actinomycin D, L-phenylalanine mustard (10, 35), DL-glyceraldehyde, and sodium oxamate (10).

We have studied the combined effect of BLEO[2] and hyperthermia on cell survival. BLEO, a complex glycopeptide, has proven valuable in the treatment of squamous cell carcinoma, lymphomas, and testicular carcinoma (4), perhaps as a result of its relative specificity for noncycling cells (2, 14, 39). However, *in vitro* cell survival studies have shown that it elicits a biphasic response for both dose (1, 2) and duration of exposure (1, 2, 39), so that its range of effective drug concentration is limited. In addition, recovery of cells of the EMT-6 tumor *in vivo* has been observed following treatment with BLEO (16), and this involves primarily noncycling cells (14).

For these reasons we became interested in different methods which might enhance BLEO toxicity and inhibit

[1] This work was supported by Grants CA-04542, CA-10372, and CA-15769.

Received April 21, 1975; accepted July 17, 1975.

[2] The abbreviation used is: BLEO, bleomycin.

the recovery of cells from BLEO damage. Studying hyperthermia in this regard, we found that in Chinese hamster cells exposed to BLEO killing proceeded much more rapidly at 43° than at 41°. This finding was difficult to explain on the basis of changes in activation energies, and it therefore suggested a qualitative difference in cell response at these temperatures (13).

We describe in this paper the response of cells to different sequences of 43° and BLEO exposure, and detail the kinetics of cell recovery from the damage produced by the combined action of these agents. Our conclusion is that inhibition of cell recovery by hyperthermia probably represents a major cause of the enhanced toxicity that we observed after the combined action of BLEO and 43° hyperthermia.

## MATERIALS AND METHODS

**Cell Culture.** HA1 cells, a pseudodiploid Chinese hamster cell line not known to be neoplastic, were regularly screened for *Mycoplasma* and grown in monolayers as previously described (15). "Unfed" cultures were obtained by seeding $10^5$ cells into 60-mm Petri dishes and were used on Day 8. The cell density was approximately $3 \times 10^5$ cells/sq cm; no significant change in cell number was observed after Day 6. The culture consisted primarily of $G_1$-like cells (15), although up to 20% of the cells may have been in $G_2$; the plating efficiency on the 8th day was approximately 60%.

Unfed cultures are sensitive to stimulation by fresh medium, and heat sensitivity varies markedly with the type of medium in which the cells are exposed (12). All experiments reported were performed with depleted medium collected from replicate unfed cultures and passed through a 0.22-$\mu$m filter prior to use.

**BLEO and Hyperthermia.** Concentrated solutions of BLEO (Blenoxane; Bristol Laboratories, Syracuse, N. Y.) were prepared in Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Santa Clara, Calif.), and dilutions were prepared in depleted medium within 6 hr of use. Unless otherwise indicated, BLEO exposures were for 1 hr, following which the cells were rinsed twice with phosphate-buffered saline.

Heat exposures were carried out in specially designed water baths which retained the temperatures ±0.1° in a 95% air-5% $CO_2$ atmosphere (12). Three different sequences of heat and drug exposure were used: (*a*) exposure at 43° without drug, followed by exposure to BLEO at 37° ("preheating"), (*b*) exposure to BLEO at 37°, followed by exposure at 43° without drug ("postheating"), and (*c*) simultaneous exposure to both agents.

Duplicate dishes were used at each experimental point. Cell survival was measured using Puck's cloning assay.

**[$^{14}$C]BLEO.** [$^{14}$C]BLEO A-2 (Nippon Kayaku Co., Ltd., Tokyo, Japan) was obtained at a specific activity of 38.59 $\mu$Ci/mg. The identical procedure for unlabeled BLEO exposure was used in the [$^{14}$C]BLEO experiments, except that 2.5 rather than 5.0 ml of the [$^{14}$C]BLEO-medium solution were added to each dish. After [$^{14}$C]BLEO exposure, cultures were rinsed 3 times with Hanks' balanced salt solution and allowed to dry. The bottoms of the plastic dishes were then punched out, and the radioactivity of the retained [$^{14}$C]BLEO was determined by a planchet-type gas-flow counter.

## RESULTS

**Dose and Time Response.**[3] The survival for unfed cells was determined for various drug concentrations at 37°, 41°, and 43° (Chart 1). The results, normalized to controls that had been exposed to the appropriate temperature in the absence of drug, show that the rate of cell killing increased appreciably between 41° and 43°. One hr of exposure at 43° in the absence of BLEO prior to drug exposure at 37° also produced a substantial degree of sensitization. In contrast, unfed cells preheated for 1 hr at 41° did not retain sensitization (data not shown).

To determine whether cytotoxicity at 43° was due to damage similar to that responsible for heat toxicity itself, cells were preheated for 1 hr at 43° and then incubated for 1 hr at 37° as a "heat repair" period (in this paper, we define repair as the increase of survival observed when cells are allowed to incubate at 37° for a period of time between treatment and the stimulation of cell proliferation by trypsin). After the heat repair period, the cells were exposed at 37° to various drug concentrations (Chart 1, Δ). Repair of heat damage under these conditions is essentially complete within 1 hr after hyperthermia. However, the degree of sensitization was unaffected by this period. Thus, it was unlikely that the combined effect was related only to the type of damage responsible for heat toxicity.

The survival of cells to a fixed dose (20 $\mu$g/ml) but variable time of BLEO exposure was measured at 37° and 43° (Chart 2). Again we observed that the biphasic part of the BLEO response was less noticeable at the higher temperature.

**Preheating Response.** To determine the dependence of sensitization on the duration of 43° exposure, we heated cells for various periods of time immediately prior to a fixed drug dose (50 $\mu$g/ml) at 37° (Chart 3) and then assayed for clonogenicity. Results were normalized to unheated control groups; thus, if no sensitization occurred, the BLEO response would coincide with the heat toxicity. However, preheating and BLEO reduced cell survival geometrically with the duration of heat exposure. At 41° we also observed slight sensitization, which developed only over a period of several hr (data not shown).

**[$^{14}$C]BLEO Uptake.** The sensitization described in the previous sections could have involved hyperthermic en-

[3] Surviving fraction is defined as:

$$\frac{\text{(Plating efficiency of experimental group)}}{\text{(Plating efficiency of untreated control)}}.$$

Surviving fraction ratio is defined as:

$$\frac{\text{(Plating efficiency of experimental group)}}{\text{(Plating efficiency of specially designated experimental group)}}.$$

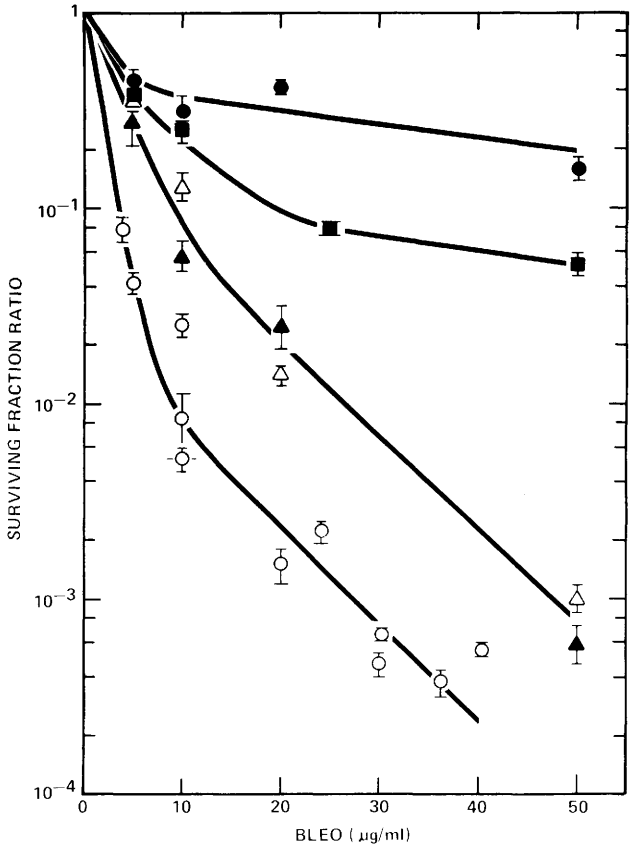


Chart 1. BLEO dose response for 1 hr of exposure at 37° (●), 41° (■), and 43° (○). One hr of preheating at 43°, followed by a period of incubation at 37°, and then by 1 hr of exposure to BLEO (37°): ▲, no 37° incubation; △, 1 hr of incubation at 37°. All values normalized to heat toxicities. *Vertical lines*, ±1 S.E. for 5 replicate dishes.

hancement of cell permeability to BLEO. We therefore reexamined the dose response experiments described in the previous section but used [$^{14}$C]BLEO. The cultures used in these labeling experiments were treated in a fashion similar to those of the corresponding survival experiments except that, after rinsing, the labeled groups were not trypsinized for the survival assay but instead were allowed to dry so that the intracellular [$^{14}$C]BLEO could be determined (Chart 4). In all groups, uptake was a linear function of drug concentration. Surprisingly, the group exposed at 37° consistently retained more drug than the groups exposed to 43°. In addition, the group preheated at 43° prior to BLEO exposure retained less drug than the group exposed simultaneously to 43° and BLEO. Therefore, we find no correlation of BLEO uptake with survival under these various conditions.

**Repair.** As previously reported (33, 38), repair of potentially lethal BLEO damage is observed with plateau phase cells (Chart 6). We first attempted to inhibit BLEO repair by heating cells to 41° immediately following a 37° BLEO exposure. However, BLEO repair occurred even during the heat "block," proceeding at 41° as rapidly as at 37° (data not shown).

In order to determine the extent of repair of the damage produced by 43° preheating and BLEO, replicate groups were incubated at 37° for 16 hr following the heat and drug exposure (Chart 3). The survival shoulder was extended by repair; however, the same final rate of cell killing was observed both with or without the repair interval, suggesting that durations of exposure at 43° in excess of about 45 min inhibited repair.

In an attempt to distinguish between sensitization and repair inhibition by 43° hyperthermia, we exposed cultures at 37° to BLEO (50 μg/ml), followed immediately by various durations of 43° exposure. Replicate dishes were subcultured to determine survival immediately after the drug-heat exposure, or after an additional 16 hr of incubation at 37° (Chart 5).

Recovery by cells exposed to heat alone was observed in this experiment, suggesting an inconsistency with the apparent lack of such repair observed in the corresponding preheating experiment (Chart 3). However, initial survival in the postheating experiment was lower than in the preheating experiment, reflecting the fact that, in the former, survival was assayed immediately after heat exposure while, in the latter, the period of drug exposure following the heat exposure provided a 1-hr repair interval for the heat controls (which were incubated in drug-free medium). The heat control results were therefore consistent with the rapid rate of repair of heat-induced damage noted in the introduction.

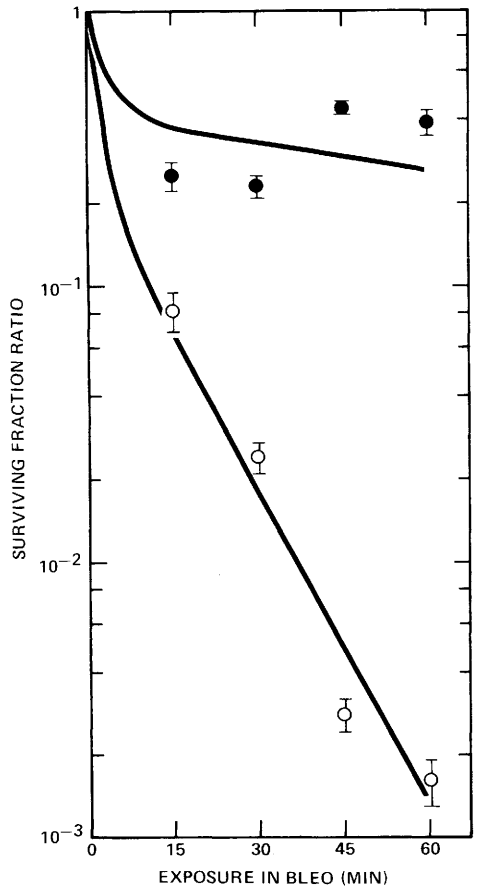


Chart 2. Time response for BLEO (20 μg/ml): 37° (●) and 43° (○). All values normalized to corresponding heat toxicities.

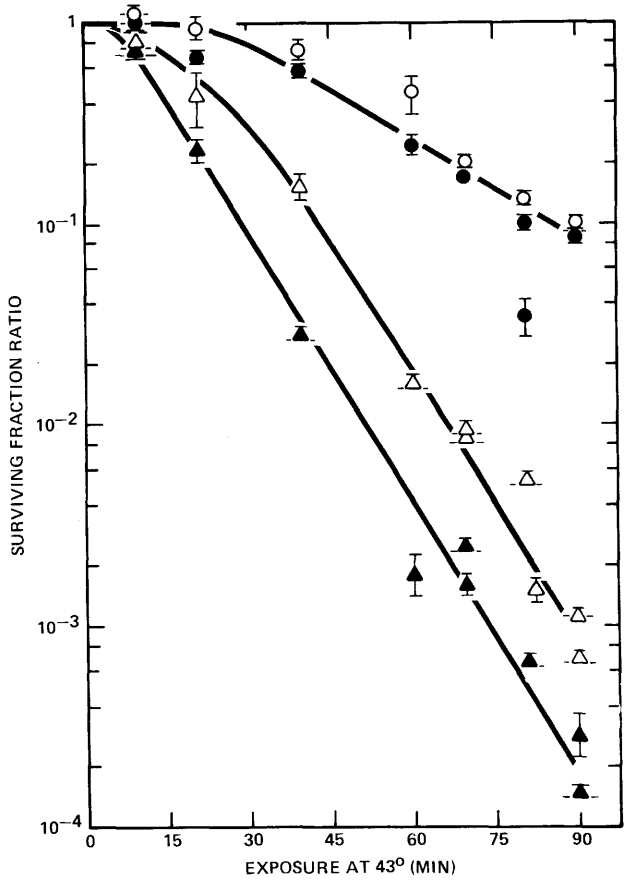


Chart 3. Response to preheating at 43°. Cells were heated at 43° for the periods of time indicated on the *abscissa* and then were exposed to BLEO (50 μg/ml) for 1 hr at 37°. Survival immediately after BLEO: ●, control (no drug); ▲, BLEO. Survival after an additional 17-hr incubation period at 37°: O, control; Δ, BLEO. Results normalized to corresponding unheated groups.

Short durations of exposure at 43° produced an additive effect on both initial BLEO survival and on survival after the repair interval, *i.e.*, the normalized heat-drug response coincided with the response to heat alone. However, postheating for periods longer than ~60 min reduced the magnitude of repair, and after 100 min of postheating, repair was essentially abolished.

To compare pre- and postheating sensitization, we determined the kinetics of repair following 40- or 80-min exposures at 43° (Chart 6). In both cases, the preheated groups sustained a substantially larger initial toxicity; however, the preheated groups rapidly recovered from the lower survival level, so that their final survival closely corresponded to the survival of the corresponding postheated cells.

## DISCUSSION

This report has considered 3 aspects involved in the combined action of heat and BLEO: (*a*) the effect of 43° on cell permeability, (*b*) the qualitative difference between 41° and 43° on cell sensitization to BLEO, and (*c*) the kinetics of repair observed after the heat and drug exposure. We now consider the importance of each factor in determining cell survival.

A simple explanation for 43° sensitization involves the possible increase of cell permeability to BLEO. Effects of hyperthermia on membrane permeability have been shown in several experimental systems (5); the suggestion that cell permeability may be a limiting factor in BLEO toxicity was made previously (7, 39) and has received support from several lines of evidence. Characterization of a BLEO-sensitive mutant strain of *Escherichia coli* correlated the phenotype with an increased rate of drug uptake (30, 43); the greater toxicity of BLEO against mouse squamous cell carcinoma *versus* mouse skin sarcoma (both induced by methylcholanthrene) was correlated with a higher rate of drug uptake by the carcinoma (although this sensitivity also paralleled reduced activity for a BLEO-inactivating enzyme) (41).

The measurements reported in this paper indicate that no gross change in cell permeability can be correlated with the toxicity produced by exposure to BLEO and 43°. However, this observation does not exclude the possibility that the change in permeability is more localized. An illustration of this point was shown in the differential effects of various polyene antibiotics on BLEO-induced inhibition of macro-

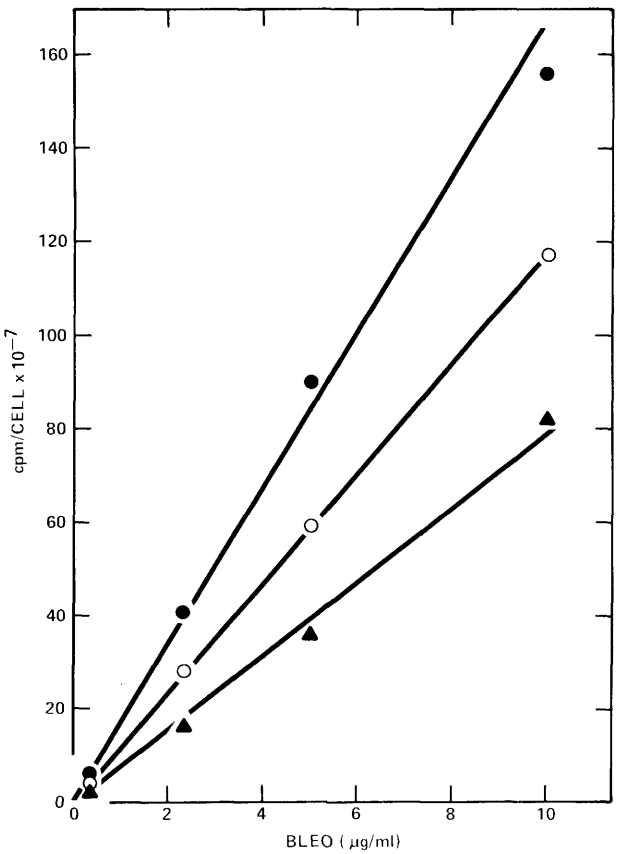


Chart 4. Dose response for [$^{14}$C]BLEO. Cells were exposed for 1 hr to 2.5-ml aliquots of various [$^{14}$C]BLEO concentrations, then were rinsed 3 times with Hanks' balanced salt solution; cpm/dish were determined with planchet-type gas flow counter. Uptake at: 37° (●), 43° (O); 1 hr preheating at 43° followed by 37° [$^{14}$C]BLEO (▲). Each *point* represents average of duplicate dishes.

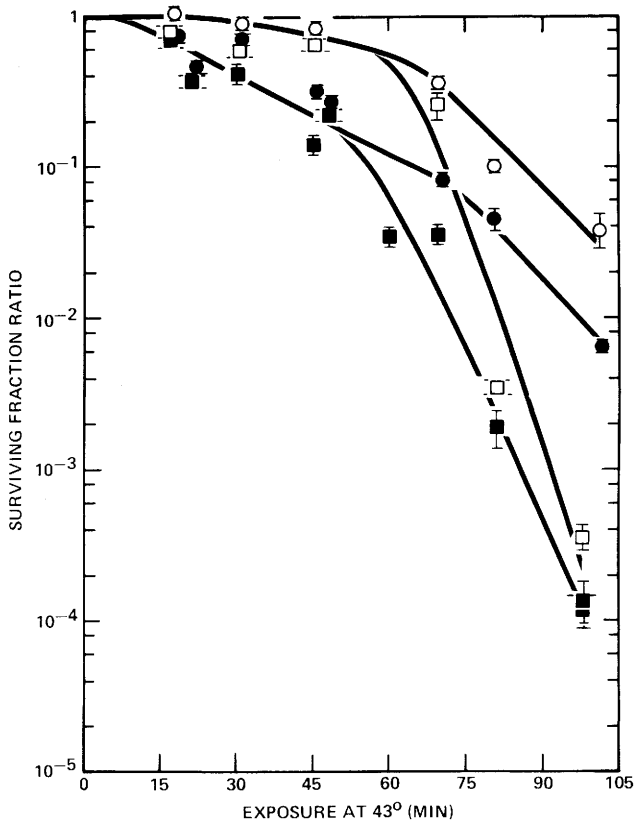


Chart 5. Response to 43° postheating. Cells were exposed to BLEO (50 μg/ml) for 1 hr at 37°, followed by 43° postheating for the durations indicated on the *abscissa*. Survival immediately after 43°: ●, control (no drug); ■, BLEO. Survival after additional 16-hr incubation period at 37°: ○, control; □, BLEO. Results normalized to corresponding unheated group.

molecular synthesis. These antibiotics all demonstrate a selective affinity for sterols in the membrane (3, 21) and, as a result, they reduce membrane integrity. Of the drugs tested, however, synergy of pentamycin and BLEO was observed (29) while no such interaction was observed with amphotericin B (22). In addition, autoradiography was recently used to suggest that the integrity of the nuclear membrane as a drug barrier might determine cell sensitivity to BLEO (8).

Perhaps the most interesting feature of the results we have described involves the qualitative difference between drug toxicity at 41° and 43°. Unlike previous reports of additive heat-drug toxicities at 42° (10, 32) or of an interaction yielding a linear $Q_{10}$ relationship, and thus no qualitative change in the nature of the lethal event(s) (18), the 43° sensitization which we observed with BLEO is of such great magnitude that when compared to 41° sensitization it suggests a novel hyperthermic effect. The possible synergistic toxicity of heat with L-erythro-α,β-dihydroxybutyraldehyde (10) and adriamycin (13) has also been observed; however, the contrasting actions of these drugs make it difficult to infer that in each case the same sensitizing lesion is involved.

The effects of hyperthermia on the response of cells to BLEO is probably due in part to the unusual mode of action of this drug. Cell killing by BLEO perhaps involves strand scission (9, 17, 26, 28), and this action is unique in that it appears to be dependent on a specific conformation of the BLEO molecule about the DNA substrate (27, 37). Although hyperthermia may increase the reaction rate, different factors related to drug action may predominate at different temperatures. The 43° preheating experiment demonstrated that sensitization involved a lesion that was not rapidly repaired (*cf.* Chart 1), while at 41° there was no evidence of the persistence of sensitizing damage. Thus, the response of cells to BLEO at 41° may primarily involve enhancement of the reaction rate for drug damage, while the response at 43° seems to depend mainly on a different sort of heat effect.

Recovery after BLEO exposure described here and elsewhere (16, 33, 38) involves a repair mechanism that is probably enzymatic (25, 38, 40), and we have seen that repair may be temporarily or permanently blocked by increasing durations at 43°. Suppose that cell survival depends on the continuous function of this repair mecha-

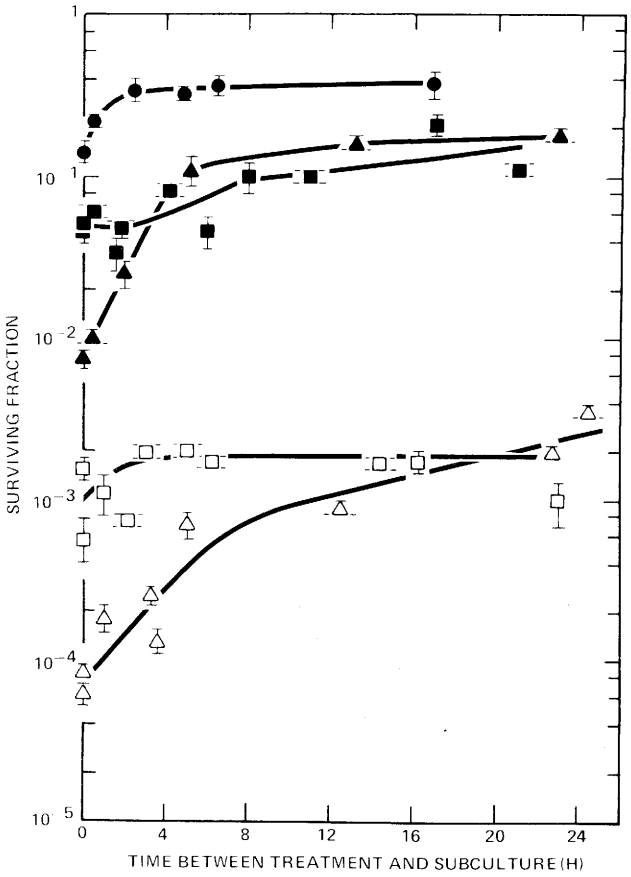


Chart 6. Effect of heat-drug sequence on the kinetics of repair. Recovery from potentially lethal damage was determined during incubation at 37° after the following treatments: ●, control (no heat); ▲, 40 min of preheating at 43° followed by BLEO exposure; △, 80 min of preheating at 43° followed by BLEO; ■, BLEO exposure followed by 40 min of postheating at 43°; □, BLEO exposure followed by 80 min of postheating at 43°, All groups were exposed to BLEO (50 μg/ml) for 1 hr at 37° exposure. *Zero hr* indicates start of 37° repair period. All results are normalized to corresponding 37° plating efficiencies.

nism during and after drug exposure; then, inhibition of the protective function of the repair mechanism at 43° would offer an explanation of the 43° sensitization. Such a mechanism cannot be determined directly from survival experiments. By definition, we measure repair as the increase in survival which follows drug exposure; the magnitude of the protective role of the repair system during exposure is difficult to assess.

However, the marked drop in survival observed in BLEO-treated cells postheated at 43° for periods of exposure longer than about 1 hr (Chart 5) may reflect the conversion of potentially lethal lesions into lethal lesions due to the hyperthermic inhibition of such a protective function. This explanation is supported by the observation that the survival level after the completion of repair does not seem to depend on the sequence of heat and drug exposure.

The effects of 43° on cell recovery from BLEO damage may be summarized by operationally defining 2 types of damage (Chart 7): (*a*) a hypersensitive response produced by preheating at 43°, where survival of stimulated cells is geometrically dependent on heat duration, but repair is efficient and relatively resistant to heat exposure, and (*b*) a threshold response produced by both heating sequences and observed clearly with 43° postheating. Increased combined cell killing occurs when the duration of 43° exceeds 1 hr. It is paralleled by the inhibition of repair activity which is nearly abolished by a 90-min exposure to 43°. As a model, this summary implies that the final survival level for both groups is determined only by the threshold response.

The relevance of our findings to cells *in vivo* is necessarily subject to the limitations of an artificial model. However, the importance of the inhibition of BLEO repair at 43° was recently emphasized by a preliminary study of the combined effect of 43° and BLEO on the survival of cells from the EMT-6 tumor treated *in vivo*. Cells from tumors excised 2 hr after drug injection showed only additive heat and drug cell killing. However, cells from tumors excised 24 hr later showed no increase in survival; the repair seen in the unheated control (from $10^{-3}$ survival to 50% at a dose of 40 mg/kg) was completely blocked by heating (G. Hahn, L. Gordon, and D. Kozak, unpublished data).

Thus what we have called thermochemotherapy may offer the potential for substantially increasing the tumor-to-normal tissue lethality of isolated tumors. What is required is the development of practical units for tumor heating *in situ* before clinical use of these findings can be seriously pursued.

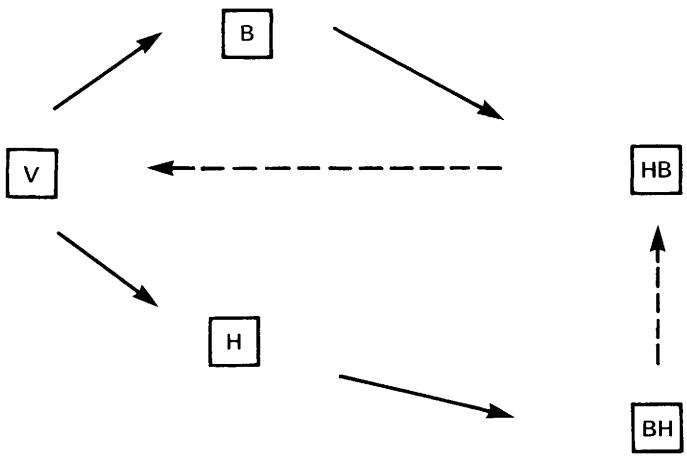


Chart 7. Summary of combined heat and drug action. Viable cells (*V*) initially enter an intermediate state immediately after exposure (→) to either 43° (*H*) or BLEO (*B*). Exposure to the other agent places cells of the 1st intermediate state into a 2nd intermediate state (*HB* or *BH*) which is unique to the sequence of agents which is used: thus, state *HB* is not equivalent to state *BH*. However, cells in state *HB* efficiently repair (⇢) hypersensitive damage and thus enter to state *BH*. In contrast, repair of threshold damage is inefficient and decreases with increasing durations of exposure at 43°.

## ACKNOWLEDGMENTS

We wish to thank Dr. T. Terasima for his assistance in obtaining [$^{14}$C]BLEO, and Terry S. Marshall for her patient and excellent help in preparing this manuscript.

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