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Induction of Thermotolerance and Sensitization in CHO Cells by Combined Hyperthermic Treatments at 40 and 43°C

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Abstract—*Chinese hamster ovary cells exposed to combined hyperthermic treatments at 40 and 43°C showed an increase or decrease in cellular sensitivity depending on the temporal order of the application of the two temperatures. Treatments at 40°C prior to hyperthermia at 43°C induced pronounced thermoresistance; the D_0 of the subsequent 43°C survival curves increased by factors of 1.1, 1.9 or 3.5 and the D_q by factors of 2.2, 2.4 or 2.5 for a 1, 3 or 5-hr pre-treatment, respectively. The magnitude of thermotolerance is fully developed by the end of pre-treatment and is not further enhanced by an incubation at 37°C following the 40°C pre-treatment. When the two thermal doses are separated by incubation at 37°C, the magnitude of thermotolerance remained constant for about 2 hr followed by a slow decline. After 24 hr at 37°C, survival is still considerably higher than expected without induced thermotolerance.*

If the order of the two hyperthermic treatments is reversed, CHO cells are rendered sensitive to 40°C, which is virtually non-lethal when it is not preceded by high hyperthermia. After hyperthermic treatments at 43°C for 30 or 60 min, inactivation at 40°C occurs exponentially, the D_0 amounting to 1.88 ± 0.03 hr and 1.49 ± 0.08 hr, respectively. This sensitization completely disappears in the course of 7 hr when the cells are incubated at 37°C after conditioning with 43°C hyperthermia.

INTRODUCTION

ALTHOUGH the effects of elevated temperatures on biological systems have been investigated for about one century, during the past 10 yr new interest has been stimulated in the use of hyperthermia as a potential anti-cancer agent (cf. reviews [1–3]). Since clinically useful protocols will depend, almost certainly, on fractioned regimens of heat alone or heat combined with other therapeutic modalities, it is necessary to study the effects of repeated hyperthermic exposures on mammalian cells or tissue systems. The problem of induction of thermotolerance is of particular concern in this regard (cf. reviews [4–8]).

Thermotolerance may be defined as a change in cellular sensitivity to succeeding thermal doses produced by an initial thermal exposure. It has been shown that thermotolerance can be induced in mammalian cells in three different ways; firstly, by heating for a short time at temperatures exceeding 43°C followed by an incubation at near-physiological temperatures [9, 10]; secondly, in the course of prolonged heating at temperatures between 41.5 and 42.5°C [5, 11]; and thirdly, by incubation at 38–41°C prior to hyperthermic treatments at 43–45°C [12–14]. By contrast, if in the latter sequence, the temporal order of application was reversed and hyperthermia at higher temperatures was followed by incubation at 38–41°C, thermosensitization was induced [12–14]. Since the modification of thermosensitivity by additional treatments at temperatures below 41°C has received only little attention up to now, it appeared worthwhile to investigate further details of this effect. Since it appears that the effects observed at 40°C are larger than those at 38, 39 or 41°C [14], in the present paper we describe the influence of treatments at 40°C on hyperthermic inactivation at 43°C.

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MATERIALS AND METHODS

The experiments were performed using Chinese hamster ovary cells (CHO) kindly

provided by Dr. W. C. Dewey, Fort Collins, U.S.A. in 1974. The cells were grown in McCoy's medium supplemented by 15% fetal calf serum and 0.01% bykomycin. Twenty-four hr after trypsinizing and reseeding 7.5×10^5 cells in 25 cm^2 Falcon plastic flasks, the cells were exposed to elevated temperatures by completely immersing the sealed flasks in water baths, maintained at the desired temperatures within ± 0.05°C. The volume of the overlying medium was 5 ml. After treatment, cells were trypsinized, counted on a Coulter counter and plated in appropriate numbers ($< 2 \times 10^4$) into Falcon plastic Petri dishes (50 mm, dia.). After 7 days, colonies were fixed, stained and counted using a projection device described earlier [15].

The methods applied were essentially the same as those used previously [14], the only difference being that trypsinization was performed immediately after the end of the last hyperthermic exposure, whereas in our previous experiments the cells were plated 4 hr before the beginning of heat treatments. This change was introduced to avoid problems of cell multiplicity occuring in experiments where treatments were to be extended up to 24 hr. Each value plotted in the graphs represents the average of 2–4 experiments, each using 4 flasks per point at 50–150 colonies per flask. Error bars represent standard deviations (S.D.).

RESULTS

Figure 1 illustrates the response of CHO cells to the graded exposure at 43°C and its modification by additional treatments at 40°C prior to or following hyperthermia at 43°C. Treatments at 43°C alone (0 hr) lead to an inactivation curve with an initial shoulder followed by an exponential slope. Since similar curves are observed after exposure to ionizing radiations we analyzed the curves in terms of D_o, D_q and D_{10} (for definition see ref. [16]) describing the slope, the width of shoulder and exposure time for 10% survival, respectively. Regression analysis of the exponential part of the 43°C curve yields $D_o = 16.9 \pm 0.7$ min, $D_q = 21.6 \pm 3.6$ min and $D_{10} = 60.6 \pm 2$ min. These values are not statistically different from those of the 43°C survival curve reported earlier [14].

Treatments at 40°C alone have a modest effect on survival. For example, after an exposure for 5 hr, the surviving fraction is still near 90%. Nevertheless, treatments at 40°C induce pronounced thermotolerance against 43°C when administered before, but provoke an enhancement in effectiveness when applied after hyperthermia at 43°C. When compared at the 10% survival level, the lengths of exposure at 43°C differ by as much as a factor of 7.4.

Regression analysis of the exponential parts of the inactivation curves yields the values of

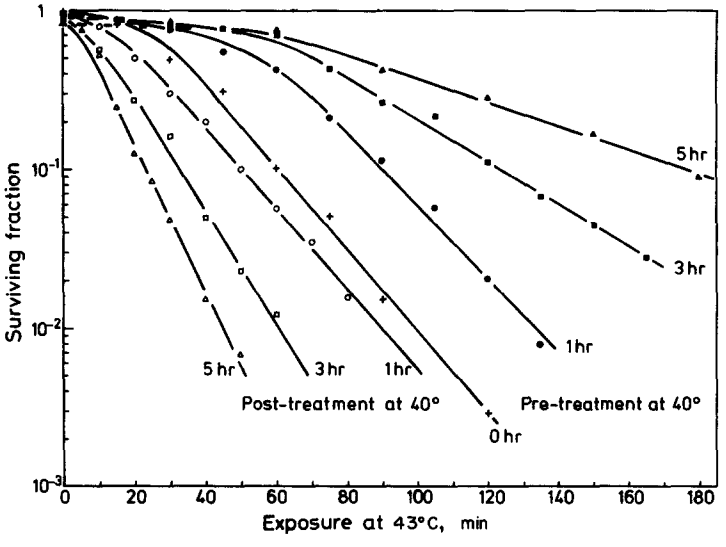

Fig. 1. Response of CHO cells to hyperthermia at 43°C and its modification by pre- and post-treatments at 40°C. + Survival after 43°C alone. (●■▲) Treatment at 40°C for 1, 3 and 5 hr, respectively, prior to graded hyperthermia at 43°C. (○□△) Treatment at 40°C for 1, 3 and 5 hr, respectively, following graded hyperthermia at 43°C. Standard deviations of the individual data points (mean 8.5% of ordinate value) have been deleted for reasons of clarity. The survival curve parameters were calculated by least-squares analysis of the points determining the linear portion and are presented in Table 1.

Table 1. Parameters D_o, D_q *and* D_{10} (±S.D.) *determined from the exponential part of the heat survival curves shown in Fig. 1*

Treatment	D_o (min)	D_q (min)	D_{10} (min)
40°C 5 hr—43°C	58.4±3.8	53.4±13.3	187.4±9.5
40°C 3 hr—43°C	32.7±1.3	51.8± 4.0	127.2±1.7
40°C 1 hr—43°C	19.0±1.0	46.9± 4.2	90.7±2.0
43°C	16.9±0.7	21.6± 3.6	60.6±2.0
43°C—40°C 1 hr	17.1±0.7	12.3± 2.6	51.6±1.0
43°C—40°C 3 hr	12.3±0.8	6.0± 4.0	34.4±1.7
43°C—40°C 5 hr	9.3±0.4	3.8± 1.4	25.2±0.7

D_o, D_q and D_{10} which are compiled in Table 1. Heat resistance acquired during 40°C pre-treatment enhanced the D_o of the 43°C survival curves by factors of 1.1, 1.9 or 3.5 and D_q by factors of 2.2, 2.4 or 2.5 for a 1-, 3- or 5-hr pre-treatment, respectively. Post-treatment for 5 hr at 40°C lowered D_o by a factor of 1.8 and D_q by 5.7. This demonstrates that thermotolerance and sensitization induced in CHO cells by additional treatments at 40°C change both D_o and D_q of the 43°C survival curve, the modification of D_o being larger for thermotolerance and that of D_q larger for sensitization.

The changes in survival by induced thermotolerance and sensitization have been investigated as a function of the duration of the treatment at 40°C up to 9 hr (Figs. 2 and 3). Here the lengths of exposure to 43°C have been chosen such as to yield surviving fractions exceeding 10^{-3}.

Figure 2 shows the increase in survival after 43°C hyperthermia caused by pre-treatments at 40°C. Hyperthermic exposures at 43°C for 90 and 120 min reduce survival to 1.55 and 0.29%, respectively. With increasing length of pre-treatment at 40°C survival increases continuously. After 40°C, 9 hr followed by 43°C, 90 min, survival is comparable with the inactivation after 40°C, 9 hr alone. That means, the thermotolerance induced by an extended pre-treatment is as high as to virtually balance the effects of a 43°C, 90 min hyperthermic treatment which alone inactivates 98.5% of the treated cells.

The kinetics of thermosensitization induced by post-treatment at 40°C is shown in Fig. 3. When hyperthermic treatments at 43°C for 30 or 60 min which reduce survival to 51 or 10.1%, respectively, are followed by exposure to 40°C, the cells surviving the first exposure are

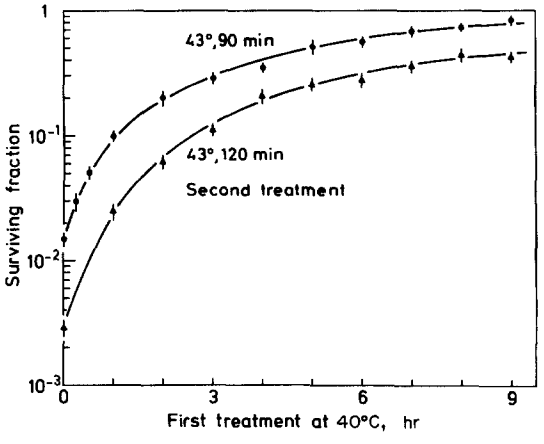

Fig. 2. Thermotolerance induced in CHO cells by pre-treatment at 40°C. Cells were exposed to 40°C for various time intervals, immediately treated at 43°C for 90 or 120 min, respectively, and assayed for colony formation.

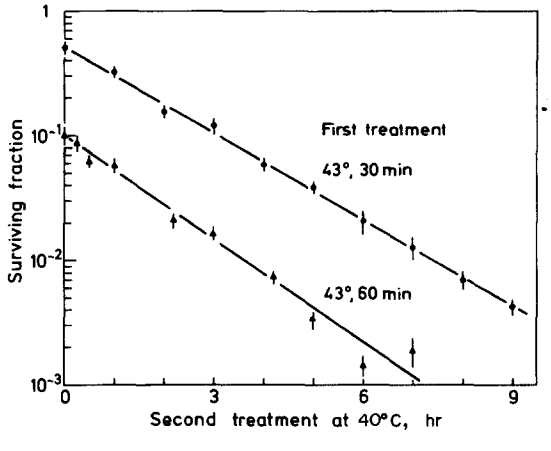

Fig. 3. Sensitization of CHO cells against hyperthermic treatments at 40°C by conditioning with 43°C hyperthermia. Cells were treated at 43°C for 30 or 60 min, respectively, immediately exposed to 40°C for various time intervals and assayed for colony formation.

inactivated exponentially with time. From the two survival curves shown in Fig. 3, values of $D_o = 1.88 \pm 0.03$ hr and $D_o = 1.49 \pm 0.08$ hr ($\pm$S.D.) are obtained by regression analysis. The difference in D_o is statistically significant on the 5% level indicating that the degree of inactivation caused by conditioning with 43°C hyperthermia affects the slope of the 40°C survival curves.

In order to investigate how long the induced thermotolerance persists in the treated cells, the cultures were maintained at 37°C for various lengths of time between exposure at 40°C and 43°C (Fig. 4). Pre-treatments at

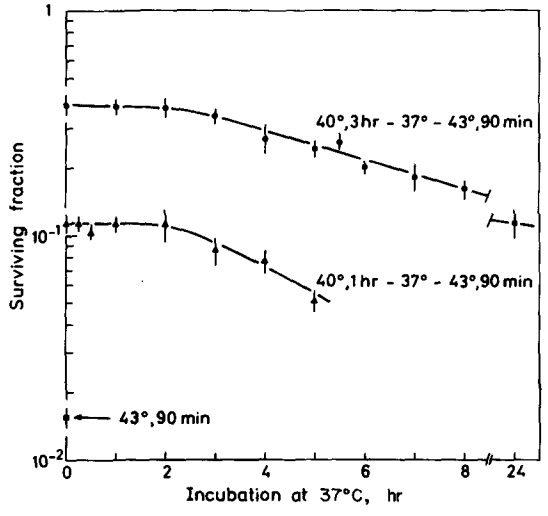

Fig. 4. Cellular surviving fraction of CHO cells exposed to a combined 40→37→43°C *hyperthermic treatment as a function of the incubation interval at* 37°C. *The cells were exposed to* 40°C *for* 1 *or* 3 hr, *respectively, followed by immersion at* 37°C *for various intervals and further exposed to* 43°C *hyperthermia for* 90 min. *The response to a single hyperthermic treatment at* 43°C *for* 90 min *is indicated by the closed square.*

40°C for 1 and 3 hr increase survival from 1.55 to 11.6 and 37.2%, respectively. These enhanced levels of survival remain virtually unchanged for about 2 hr after the end of pretreatment followed by a slow decline. When incubation at 37°C was extended to 24 hr between the 40°C, 3 hr and 43°C, 90 min hyperthermic treatments, survival was still 11.3 ± 1.7% which is considerably higher than found after 43°C, 90 min alone, thus indicating that the acquired thermotolerance persists, at least to a certain degree, for more than one day.

If 43°C hyperthermia is separated from 40°C post-treatment by incubation at 37°C (Fig. 5) the surviving fraction increases continuously and reaches the level of non-

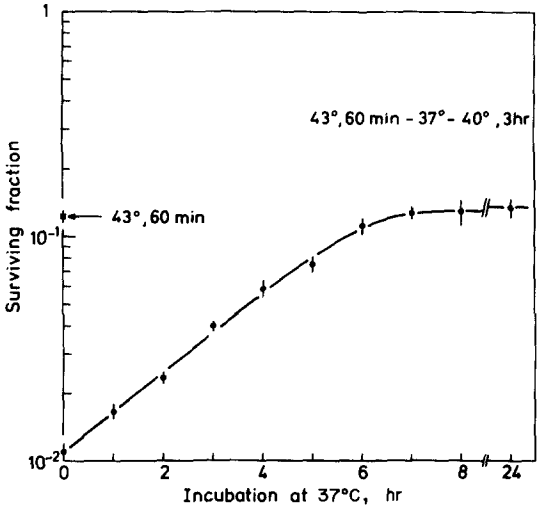

Fig. 5. Cellular surviving fraction of CHO cells exposed to a combined 43→37→40°C *hyperthermic treatment as a function of the incubation interval at* 37°C. *The cells were exposed to* 43°C *for* 60 min *followed by immersion at* 37°C *for various intervals and further exposed to* 40°C *hyperthermia for* 3 hr. *The response to a single hyperthermic treatment at* 43°C *for* 60 min *is indicated by the closed square.*

sensitized cells after about 7 hr. These findings indicate that the enhanced cell killing shown in Fig. 3 is reduced with increased intervals between the two hyperthermic treatments. No further recovery was seen when the intervals were extended to 24 hr. This indicates that relatively short treatments at 43°C induce certain types of latent damage which render the cells sensitive to subsequent treatments at 40°C. This sensitization disappears in the course of 7 hr.

DISCUSSION

The present report shows that the response of CHO cells to hyperthermic treatments at 43°C is strongly modified by additional exposure to 40°C in such a way that pretreatment induces thermotolerance whereas post-treatment enhances thermosensitivity. Survival observed after hyperthermia at 43°C is increased by factors up to 150 by pretreatments for 9 hr at 40 C (Fig. 2) whereas postreatments for 9 hr at 40°C lead to a decrease in surviving fractions by factors up to 100 (Fig. 3).

Thermotolerance induced in CHO cells by treatment at 40°C prior to hyperthermia at 43°C is considerably higher than that observed after combining 40 and 45°C. Henle and Leeper [12] found that survival of CHO cells, exposed at 45°C for 10 or 20 min, was enhanced by a factor of 1.5 when pre-

treatment at 40°C was extended for up to 7 hr. Henle *et al.* [13] showed that incubation at 40°C prior to 45°C hyperthermia only marginally altered D_o of the heat survival curves, but enhanced the D_q from 8.3 min at 45°C to 17.8, 18.2 or 22.8 min at 45°C for a 2-, 4- or 7-hr pre-treatment, respectively. In contrast, when a 40°C pre-treatment is combined with 43°C hyperthermia, the slope of the heat response curves at 43°C is altered even more strongly than is the shoulder width (cf. Fig. 1 and Table 1).

Thermotolerance induced in CHO cells by pre-treatment at 40°C is maximally developed by the end of the pre-treatment and is not further increased by a following incubation at 37°C (Fig. 4). In contrast, thermotolerance induced by fractionated exposure at higher temperatures (>43°C) requires several hours of incubation at 37°C to develop. For instance, following a conditioning heat treatment of 10 min at 45°C, the D_o of the CHO hyperthermia survival curve reached its maximum value when the samples were incubated at 37°C for 8 hr [13]. HeLa cells exposed to two 44°C, 1-hr thermal doses showed maximal thermotolerance after an incubation period at 37°C for 5 hr [17]. *In vivo* the time interval required for the development of thermotolerance seems to be longer than in cell cultures. Crile [18] investigated acquired heat resistance both in normal tissue of the feet of mice and in implanted sarcoma 180 tumours and found maximal resistance when preheating for 30 min at 44°C and the following hyperthermic treatment at the same temperature were separated by a fractionation interval of one day. A detailed investigation of induced thermal resistance has recently been made *in vivo* using necrosis of the mouse ear as the end point [19]. Even 2 min heating at 43.5°C induced significant thermal tolerance. The maximum effect occurred when the second heat doses were given 48 hr after preheating at 43.5°C for 40 min [19].

Induced thermal resistance persists for 1–3 days in cultured cells. This has been shown for several cell lines including CHO [10, 13], V-79 [20] and HeLa [17, 21] treated at temperatures of 42.5°C or above. The results of the present study suggest that thermotolerance induced by a 40°C pre-treatment persists for a comparable length of time (Fig. 4) although, in contrast to thermotolerance induced by higher temperatures, it does not require incubation at 37°C for development (see above).

Sensitization was observed when cells after conditioning with 43°C hyperthermia were treated at 40°C (cf. Fig. 3) which is virtually nonlethal for CHO cells when it is not preceded by high hyperthermia. Henle and Leeper [12, 22] reported that when cells were heated at 45°C prior to treatment at 40°C, survival showed a fast decrease during the first hour at 40°C followed by an exponential decrease. Our results obtained by combining 43°C and 40°C do not show a fast initial decline (Fig. 3) and, furthermore, the slope of the 40°C survival curves (Fig. 3) is steeper than found after a 45→40° sequence [12, 22].

Sensitization was also observed for V-79 cells when a treatment at 44°C for 15 min was followed by a graded exposure to 42°C [23]. When the two heat treatments were separated by an incubation at 37°C, the surviving fraction initially showed an exponential increase and reached a constant level (nearly the additive surviving fraction of the two single hyperthermic treatments) when an interval of time at 37°C exceeding 6–7 hr was interposed between the two exposures [23]. Thus, the recovery kinetics observed after the combination of 44→42°C was very much the same as found in the present study for 43°C combined with 40°C (Fig. 5). These results may give further support to the interpretation that hyperthermic sublethal damage may at 40°C be converted to lethal damage [22].

The understanding of thermotolerance and sensitization may be critical to the application of hyperthermia to cancer therapy. Even relatively small differences in the time course or in the magnitude of induced thermotolerance or sensitization between normal and malignant cells could affect therapeutic ratios in response to fractions of hyperthermia alone or combined with other therapeutic modalities, such as radiation or chemotherapy. Whether an increased therapeutic ratio between tumour and normal cells would actually result from specific fractionation schemes of these combined modalities remains to be determined.

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REFERENCES

1. F. Dietzel, *Tumor und Temperatur.* Urban & Schwarzenberg, München (1975).
2. Proceedings of the International Symposium on Cancer Therapy by Hyperthermia and Radiation. Washington, D.C. April 28–30 (1975). American College of Radiology.
3. C. Streffer, *Cancer Therapy by Hyperthermia and Radiation.* Urban & Schwarzenberg, München (1978).
4. B. Bronk, Thermal potentiation of mammalian cell killing: clues for understanding and potential for tumor therapy. *Advanc. Radiat. Biol.* **6,** 267 (1976).
5. W. C. Dewey, L. E. Hopwood, S. A. Sapareto and L. E. Gerweck, Cellular responses to combinations of hyperthermia and radiation. *Radiology* **123,** 463 (1977).
6. W. G. Connor, E. W. Gerner, R. C. Miller and M. L. M. Boone, Prospects for hyperthermia in human cancer therapy. II. Implications of biological and physical data for applications of hyperthermia to man. *Radiology* **123,** 497 (1977).
7. K. J. Henle and L. A. Dethlefsen, Heat fractionation and thermotolerance: a review. *Cancer Res.* **38,** 1843 (1978).
8. S. B. Field and N. M. Bleehen, Hyperthermia in the treatment of cancer. *Cancer Treat. Rev.* **6,** 63 (1979).
9. E. W. Gerner and M. J. Schneider, Induced thermal resistance in HeLa cells. *Nature (Lond.)* **256,** 500 (1975).
10. K. J. Henle and D. B. Leeper, Interaction of hyperthermia and radiation in CHO cells: recovery kinetics. *Radiat. Res.* **66,** 505 (1976).
11. S. A. Sapareto, L. E. Hopwood, W. C. Dewey, M. R. Raju and J. W. Gray, Effects of hyperthermia on survival and progression of Chinese hamster ovary cells. *Cancer Res.* **38,** 393 (1978).
12. K. J. Henle and D. B. Leeper, Combinations of hyperthermia (40°, 45°C) with radiation. *Radiology* **121,** 451 (1976).
13. K. J. Henle, J. E. Karamuz and D. B. Leeper, Induction of thermotolerance in Chinese hamster ovary cells by high (45°) and low (40°) hyperthermia. *Cancer Res.* **38,** 570 (1978).
14. D. S. Joshi and H. Jung, Thermotolerance and sensitization induced in CHO cells by fractionated hyperthermic treatments at 38–45°C. *Europ. J. Cancer* **15,** 345 (1979).
15. H. Jung, Eine einfache Anordnung zum Auszählen von Zellkolonien. *Leitz-Mitt. Wiss. u. Techn.* **7,** 102 (1978).
16. E. J. Hall, *Radiology for the Radiologist.* Harper & Row, Hagerstown (1973).
17. E. W. Gerner, R. Boone, W. G. Connor, J. A. Hicks and M. L. M. Boone, A transient thermotolerant survival response produce by single thermal doses in HeLa cells. *Cancer Res.* **36,** 1035 (1976).
18. G. Crile, The effects of heat and radiation on cancers implanted on the feet of mice. *Cancer Res.* **23,** 372 (1963).
19. M. P. Law, P. G. Goultas and S. B. Field, Induced thermal resistance in the mouse ear. *Brit. J. Radiol.* **52,** 308 (1979).
20. L. Harisiadis, D. I. Sung and E. J. Hall, Thermal tolerance and repair of thermal damage by cultured cells. *Radiology* **123,** 505 (1977).
21. J. T. Leith, R. C. Miller, E. W. Gerner and M. L. M. Boone, Hyperthermic potentiation. Biological aspects and applications to radiation therapy. *Cancer (Philad.)* **39,** 766 (1977).
22. K. J. Henle and D. B. Leeper, Interaction of sublethal and potentially lethal 45°-hyperthermia and radiation damage at 0, 20, 37 or 40°C. *Europ. J. Cancer* **15,** 1387 (1979).
23. J. Miyakoshi, M. Ikebuchi, M. Furukawa, K. Yamagata, T. Sugahara and E. Kano, Combined effects of X irradiation and hyperthermia (42 and 44°C) on Chinese hamster V-79 cells *in vitro. Radiat. Res.* **79,** 77 (1979).