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Thermotolerance and Sensitization Induced in CHO Cells by Fractionated Hyperthermic Treatments at 38°–45°C*

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Abstract—*The response of cultured Chinese hamster ovary (CHO) fibroblast cells to hyperthermia at 43° and 45°C and its modification by additional hyperthermic treatments at temperatures from 38° to 45°C were determined. Treatment of CHO cells at temperatures below 43°C prior to exposure at 43° or 45°C induced pronounced thermal resistance, with an increase in surviving fraction by factors up to 20. In contrast, hyperthermia at lower temperatures enhanced the effectiveness of hyperthermia at 43° or 45°C if the order of application was reversed. Pre- or post-incubation at 44° or 45°C enhanced the effectiveness of hyperthermia at 43°C, irrespective of the order of application.*

INTRODUCTION

THERE is a resurgence of interest in hyperthermia as a possible means of treating cancer. Its application either alone or as an adjunct to conventional radiotherapy is being contemplated by several clinicians, although relatively few clinical trials have been reported to date (for review see [1]).

For therapeutic application of hyperthermia, more experimental data are required especially since there are now increasing numbers of reports on induction of thermotolerance in cells during fractionated or prolonged heat treatments [2–7]. In addition to the induction of thermotolerance *per se*, various other factors seem to contribute to the phenomenon observed, such as recovery from sublethal thermal damage or redistribution of cells within cell cycle. Clinical application of hyperthermia also entails consideration of temperature at which heat is to be applied. The significant rate of cell kill per unit time of heat application is achieved at temperatures in excess of 42°C. Such severe treatments, however, may not be tolerated well by human beings. On the other hand, the treatments below 42°C, although well within the tolerance limits may not achieve significant cell kill.

In view of this it is worthwhile to explore the possibility of applying heat in successive fractions of mild and severe hyperthermia. The effect of successive fractions at temperatures above and below 42°C should be different from the additive effect of the two treatments since different mechanisms of cell inactivation have been shown to operate in these ranges of temperature [8, 9]. The optimum application of such fractionated heat treatments in terms of temperature, duration and sequence can have significant clinical implication.

In the present work we investigated effects of fractionated hyperthermic treatments on cultured Chinese hamster ovary (CHO) fibroblasts. The rationale of the experiments was to apply non-lethal, moderately lethal and lethal hyperthermic treatments (referring to survival levels of more than 95%, more than 80% or less than 50%, respectively) for fixed time intervals and to determine the modification of cellular response to a preceding or following graded exposure at 43° or 45°C. Incubations for fixed duration were performed at temperatures ranging from 38° to 45°C in order to investigate whether temperatures

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below and above 43°C cause different effects so far as enhancement or decrease in the effectiveness of hyperthermia at 43° or 45°C are concerned.

MATERIALS AND METHODS

Asynchronous cultures of CHO cells, grown and maintained in McCoy's medium supplemented by 15% fetal calf serum were used for these investigations. Cells in logarithmic phase of growth were trypsinised and plated in 25 cm^2 Falcon flasks, 4 hr before the commencement of heat treatments. The flasks containing 2.5 ml of medium each, were sealed and completely immersed in the water baths, maintained at the desired temperatures within ± 0.05°C. After the treatments, the flasks were returned to the CO_2 incubator on addition of 2.5 ml of fresh medium to each of the flasks. The colonies, stained with crystal-violet were counted 8 days after plating.

RESULTS

Hyperthermia at 43°C

Figure 1 illustrates the response of CHO cells to the graded exposure at 43°C and its modification by additional treatments at 41°C

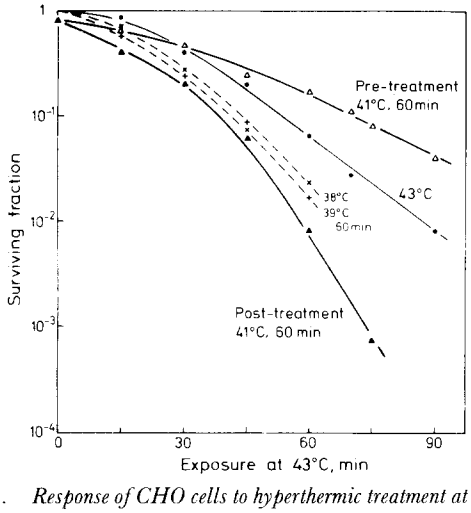

Fig. 1. Response of CHO cells to hyperthermic treatment at 43°C *and its modification by pre- and post-treatments for* 60 min *at lower temperatures.* ● *Survival after* 43°C *alone,* △ 41°C *for* 60 min →43°C, ▲ 43°→41°C, ×43°→38°C, +43°→39°C.

prior to or following incubation at higher temperature. It is evident that moderately lethal treatment at 41°C which by itself decreases the surviving fraction to 0.83, induces a pronounced tolerance to hyperthermia at 43°C when added before, but causes greater enhancement in effectiveness of hyperthermia at 43°C when added after such treatments. The data read off the curves ($D^o = 14.5$ min, $D^q = 20$ min for 43°C alone; $D_o = 22.5$ min, $D_q = 22$ min for 41°→43°C; and $D_o = 7.0$ min, $D_q = 27$ min for 43°→41°C) indicate that decrease and enhancement in the effectiveness are more due to changes in slopes than in shoulder widths. Post-incubation for 1 hr at still lower temperatures such as 38° or 39°C was found to induce similar enhancement but was less effective than 41°C in enhancing lethality of hyperthermia at higher temperatures.

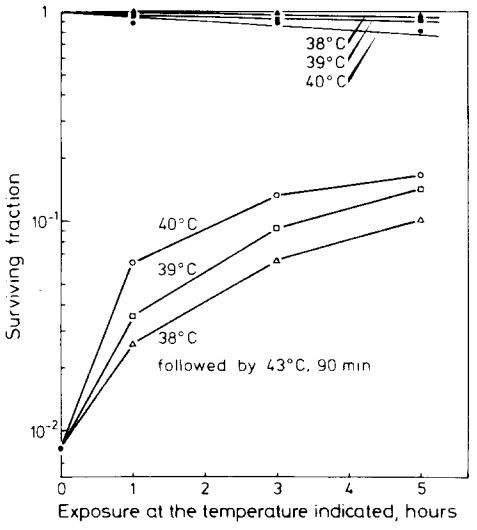

Fig. 2. Induction of thermotolerance in CHO cells against hyperthermia at 43°C *for* 90 min *by pre-incubation at lower temperatures: effect of incubation at* 38°–40°C *for various durations of incubation.* ▲, ■, ●, *Inactivation by hyperthermia at* 38°, 39° *and* 40°C *alone, respectively.* △ □ ○, *Effect of pre-treatments at* 38°, 39° *and* 40°C, *respectively, followed by treatment at* 43°C *for* 90 min, *as a function of duration of pre-treatments.*

Figure 2 illustrates the degree of thermotolerance as a function of temperature and duration of hyperthermia below 41°C that precedes 90 min of heat application at 43°C. With 1 hr of pre-treatment at 38°, 39° or 40°C, surviving fraction after heat exposure at 43°C is increased by factors of 3.2, 4.3 and 7.7, respectively. Since treatment at 41°C for 1 hr increases the corresponding survival by a factor of 5 (Fig. 1), this temperature appears to be less efficient for induction of thermotolerance than 40°C. With further increase in incubation time up to 5 hr at lower temperatures, there is gradual increase in the degree of thermotolerance induced. Pre-incubation for 5 hr at 38°, 39° or 40°C increases the survival at higher temperature by factors up to 12, 17 or even 20, respectively (Fig. 2, lower part), although these treatments by themselves inactivate less than 20% of the cells exposed (Fig. 2, upper part).

If non-lethal or moderately lethal treatments were applied at temperatures above

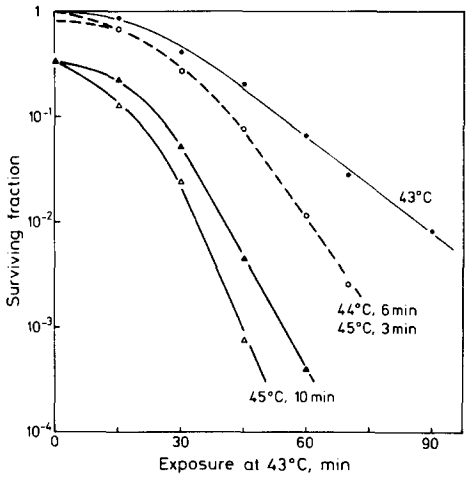

Fig. 3. Response of CHO cells to hyperthermic treatment at 43°C *and its modification by pre- and post-treatment at higher temperatures.* ● *Survival after* 43°C *alone,* △ 45°C *for* 10 min→43°C, ▲ 43°→45°C *for* 10 min, ○ *Mean of four experiments:* 44°C *for* 6 min→43°C, 43°→44°C *for* 6 min; 45°C *for* 3 min→43°C, 43°→45°C *for* 3 min.

43°C, they also modify the response of CHO cells to graded exposures at 43°C. As shown in Fig. 3, treatments at 44°C for 6 min and at 45°C for 3 min enhance the sensitivity of CHO cells to hyperthermia at 43°C to a similar extent. However, in marked contrast to the effects of hyperthermic treatments below 43°C, there is no induction of thermotolerance when such treatments precede heat application at 43°C. The enhancement of effectiveness of hyperthermia at 43°C was independent of the order of application of two heat fractions. A single curve is drawn for the effects of hyperthermia at 44° and 45°C since the respective survival curves are quite close to one another. The results thus suggest that it is not just the degree of lethality that matters for the induction of thermotolerance but the range of temperature in which pretreatment is administered.

If a lethal treatment of 10 min at 45°C administered either before or after the graded treatment at 43°C, the corresponding survival curves are shifted to lower survival level with shoulder widths (D_q: 18 and 19 min for pre- and post-treatments, respectively) being similar to that of inactivation curve at 43°C alone (D_q=20 min), but with decreased slopes (D_o: 4.6 min and 6.2 min for pre- and post-treatments as compared to 14.5 min without such treatments).

Hyperthermia at 45°C

Because of the interaction observed between non-lethal and lethal lesions induced at 45° and 43°C (Fig. 3), influence of various pre- and post-treatments was investigated on the

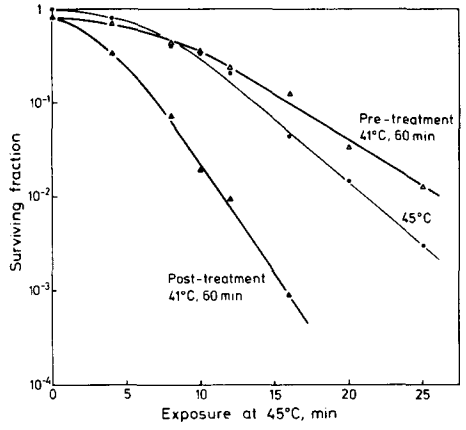

Fig. 4. Response of CHO cells to hyperthermic treatment at 45°C *and its modification by pre- and post-treatments at* 41°C *for* 60 min.

cellular inactivation at 45°C. As is evident from Fig. 4, the results are essentially similar to those obtained in case of graded exposure of CHO cells to hyperthermia at 43°C. The effectiveness of hyperthermia diminishes if 1 hr of heat application precedes incubation at higher temperature but is markedly enhanced if treatment at 41°C is performed subsequently. The data read off the curves are the following: D_q=6.1 min, D_o=3.2 min for treatment at 45°C alone: D_q=6.5 min, D_o=4.4 min for 41°→45°C; and D_q=3.1 min, D_o=1.9 min for 45°→41°C. Lethal pre- or post-treatments at 43°C for 45 min (Fig. 5) which by itself reduces survival to 33%, merely shifts the dose–response curve at 45°C with little influence on the slope of the curves.

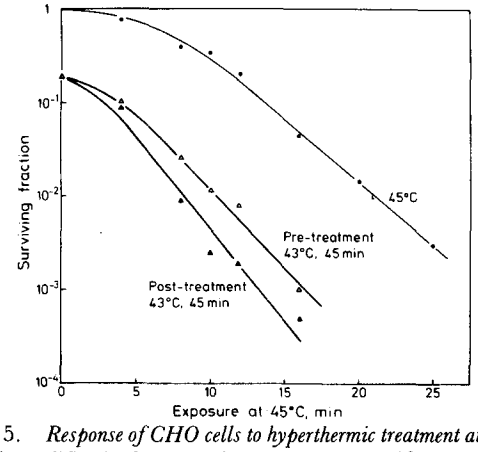

Fig. 5. Response of CHO cells to hyperthermic treatment at 45°C *and its modification by pre- and post-treatments at* 43°C *for* 45 min.

The results clearly show that exposure of CHO cells to mild hyperthermia at temperatures below 42°C prior to severe hyperthermic treatments at 43° or 45°C induces pronounced tolerance to such thermal treatments. In contrast, incubation at lower temperatures after severe hyperthermic treatments enhances the effectiveness of treatments at higher

temperatures. Such an enhancement of effectiveness of hyperthermia at 43°C is also observed for moderately lethal as well as non-lethal treatments administered at temperatures in excess of 43°C, irrespective of the order of application of the two heat fractions. At the intermediate temperature of 43°C neither decrease nor enhancement is observed to any appreciable extent.

DISCUSSION

The present report, for the first time, indicates that hyperthermic temperatures as low as 38° or 39°C modify the response of mammalian cells to elevated temperatures such that pre-treatment induces thermotolerance whereas post-treatment enhances the response.

Although several workers have reported induction of thermotolerance both *in vivo* and *in vitro*, they have used pre-treatment at 40° or 42°C [5, 9]. In some cases cultures were returned to 37°C after initial heat application for developing tolerance to subsequent hyperthermia [7]. Henle and Leeper [10] have reported that pre-treating CHO cells at 40°C for several hours enhanced survival after exposure at 45°C for 10 and 20 min by a factor of 1.5. Our results indicate a much higher degree of thermotolerance for hyperthermia at 43°C since the survival levels are increased by factors up to 20 by pre-treatments of 5 hr at 40°C (Fig. 2).

An interesting aspect of the results presented here is the ability of mild hyperthermia at 38°–41°C to modulate the effect of heat treatment at 43°C, depending on being applied before or after such treatment. On the other hand, non-lethal or moderately lethal hyperthermia, when applied above 43°C, only enhances the effectiveness of the heat treatment at 43°C.

The molecular and cellular mechanisms of modification of hyperthermic cell inactivation by addition of non-lethal or moderately lethal hyperthermia are not known at present. As for the thermotolerance induced by pre-incubation at temperatures slightly in excess of 37°C, this cannot be attributed to the redistribution of cells within the cell cycle due to such pretreatment. Data to be presented elsewhere [11] show that the percentages of cells in various phases as determined by flow cytophotometry [12] remain unchanged during 4 hr incubation at 40°C and below. By contrast, treatments at 42°C were shown to change the rate of progression of CHO cells [13] and CH-V79 cells [14] by induction of blockages in S and G_2+M phases. Enhanced effectiveness of hyperthermia due to subsequent mild hyperthermia on the other hand may be caused either by impairment of the repair of sublethal thermal damage or by conversion of sublethal lesions into lethal lesions. Nevertheless, it appears highly interesting that temperatures only slightly higher than normal can cause marked changes in the thermosensitivity of mammalian cells.

There is now increasing evidence that the thermal lesions induced above and below 43°C differ from each other as has been pointed out in 1976 [15]. Dewey *et al.* [9] have found that the Arrhenius plot for hyperthermic killing of CHO cells changes near 43°C. Similar findings have been reported for the response of a fibrosarcoma *in vivo* [16]. Hyperthermic treatments at temperatures below 43°C both during and after X-irradiation mostly increases lethal damage *in vitro* [8, 9, 17]. The response of cultured mammalian cells to some drugs is significantly enhanced by heat application above 42°C as compared to treatments below 42°C [18, 19]. Our results show that although non-lethal or moderately lethal hyperthermia below 43°C cause thermotolerance if applied before incubation at 43°C, such mild treatment at 44° or 45°C enhances cell killing by subsequent treatment at 43°C. This substantiates inference that the thermal lesions above and below 43°C differ from each other.

It is perhaps of interest to note the similarity in modification of cellular response to X-rays and to hyperthermia at 43°C by heat application at 45°C. In both the cases, prior application is more effective than one that follows irradiation [8, 9, 20] or heat application at 43°C (cf. Fig. 3). These results tend to support the view that mechanisms of cell inactivation at 43°C and above bear some similarities to mechanisms of X-irradiation induced cell inactivation.

The degree of thermotolerance induced by 1 hr of pre-incubation increases with the increases in temperature from 38° to 40°C. Further increase in temperature to 41°C, however, results in decrease in the degree of thermotolerance to the subsequent hyperthermia (Figs. 1 and 2). This may perhaps be related to the acquisition of lethal properties of thermal lesions at 41°C. Interestingly enough, if hyperthermia is applied in three successive fractions: 41°C (1 hr)→43°C (90 min)→41°C (1 hr), the observed survival

is less than expected on the basis of mere addition of three individual fractions. In other words, the results could be explained on the basis of (41°→43°C)→41°C rather than 41°→(43°→41°C) [11].

Our findings may have some significance for the clinical application of hyperthermia. If the heat application is not uniform over the entire volume of tumour, some regions may be warmed up to only 38°–40°C. This can induce tolerance to subsequent hyperthermic treatments. Mild treatments on the other hand can be deliberately used to enhance or diminish the effectiveness of severe heat treatments. Hyperthermia at temperatures above 43°C can be used where induction of thermotolerance is not desired. Since prolonged exposure at 43°C often causes severe skin damage [21], the possibility should be explored for combination of hyperthermia at 43°C for short duration followed by treatments at temperatures below 41°C. Such a schedule may afford a possibility for reduction of side effects without concomitant loss of therapeutic efficacy.

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