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Interaction of Sublethal and Potentially Lethal 45°-Hyperthermia and Radiation Damage at 0, 20, 37 or 40°C*†

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Abstract—*The interaction of hyperthermia damage and radiation damage has most often been studied under conditions which favoured repair of sublethal damage. In the present study the interaction of* 45°C-*hyperthermia damage and radiation damage on the survival of Chinese hamster ovary (CHO) cells was determined at temperatures of* 0–4°, 20° *and* 40°C. *Subphysiological temperatures inhibited to various degrees both the repair of sublethal radiation and sublethal* 45°C-*hyperthermia damage while incubation at* 40°C *apparently led to the conversion of sublethal* 45°C-*hyperthermia lesions to lethal lesions. Potentially lethal hyperthermia damage (H-PLD) was repaired at* 0–4° *and* 20°C; *whereas the repair of potentially lethal radiation damage (X-PLD) occurred at* 20°C, *but not at* 0–4° *or* 40°C.

The interaction of 45°C-*hyperthermia and radiation damage at temperatures of* 0–4°, 20° *and* 40°C *represented a super-position of the separate effects of the two treatment modalities separately combined with incubation at* 0–4°, 20° *or* 40°C. *This suggests that ionizing radiation and hyperthermia are affecting distinct targets and that H-PLD and X-PLD do not interact.*

INTRODUCTION

MAMMALIAN cells are capable of surviving incubation at non-physiological temperatures for limited periods. Subphysiological temperatures as low as 0°C are lethal to Chinese hamster V79 cells only after an exposure of several days [1]. At supraphysiological temperatures up to approximately 40°C many enzyme reactions are accelerated without loss of cell survival, while at temperatures above 40°C virtually all phases of metabolism become inhibited [2, 3] due to both reversible and irreversible enzyme inactivation [3, 4]. When cells are injured, either by radiation or hyperthermia, cell survival could become dependent on the efficiency of either repair or fixation processes [4–7]. Non-physiological, but sublethal, temperatures may be used to modulate these processes and permit the altered repair or fixation of damage to be assayed in terms of cell survival.

The combination of therapeutic modalities with sublethal hypo- or hyperthermia may also have practical applications. For example, the interaction of 45°C-hyperthermia damage and radiation damage is greatly potentiated when the two treatments are combined with incubation at 40°C [8, 9]. This may represent yet another therapeutic approach to the treatment of tumors by combined hyperthermia and radiation [8, 9]. The present report describes how the interaction of 45°C-hyperthermia damage and X-radiation damage in cultured Chinese hamster cells is modified by incubation at 0–4°, 20° and 40°C.

The terminology used for interaction is that taken from radiation studies. Sublethal damage repair is operationally defined as the increase in cell survival when a single treatment is given in two fractions separated by a given recovery period [7, 10]. Similarly, potentially lethal damage repair is defined by the

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increase in cell survival when cells are maintained under non-physiological conditions (i.e., 0° and 20°C incubation) after heating and/or irradiation [7, 11].

MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were propagated in modified McCoy's 5a medium (Grand Island Biological Co.) supplemented with 10% calf and 5% fetal calf sera (Flow Laboratories). Stock cultures were monitored routinely for the absence of mycoplasma by the method of Levine [12]. Cells from suspension cultures were inoculated in appropriate numbers into 25 cm^2 Falcon plastic flasks. About 18 hr later the flasks were sealed, and after determination of cellular multiplicity, the exponentially growing cells were heated and/or irradiated. Following 6–8 days of undisturbed growth at 37°C, the visible macroscopic colonies were fixed, stained and scored for a determination of single cell survival. Each value plotted in the graphs represents the average of 2–4 experiments, each employing 4–6 flasks per point at 50–300 colonies per flask unless otherwise indicated. Standard errors are shown, unless they were smaller than the experimental point.

All treatment temperatures were obtained by horizontal submersion of the culture flasks and equilibration with precision-controlled water baths (±0.05°C). The temperature of the medium overlying the cells rose to within 0.2°C of the temperature of the water bath by 3 min [13, 14]. Temperatures were monitored by a mercury thermometer scaled in tenth degrees from 25°–55°C (ASTM 64C, Fisher Scientific Co.) and calibrated against a thermistor directly traceable to the Natural Bureau of Standards (appreciation to Dr. Tom Cetas, Div. of Radiation Oncology, University of Arizona, Tucson, U.S.A.). For temperatures of 0°–4°C flasks were submerged in an ice bath. The pH of medium in flasks equilibrated to 20°C or 0°–4°C decreased by 0.11 and 0.19 pH units, respectively from the pH value of 7.4 at 37°C.

The conditions of X-irradiation were 37°C, 250 kVcp, 15 mA, 2mm Al added filtration, SSD of 60 cm, and a dose rate of 150 rad/min. The dose rate was calculated from ionization measurements with a Victoreen R-meter, and checked by LiF thermoluminescent dosimetry. Combined treatments with time=0 hr were sequential, with approximately 1 min between treatments.

RESULTS

45°C-*Hyperthermia*

Long-term incubation of CHO cells at 20° or 0–4°C reduced plating efficiency exponentially with time at a rate of −0.006 per hr, or −0.015 per hr, respectively (Fig. 1). A 7 hr incubation period at either 20° or 0°–4°C reduced the cellular plating efficiency by only 4 or 10%, respectively, and corrections for hypothermic cell killing were not made in subsequent analyses. In contrast, incubation at 40°C for up to 75 hr did not reduce cellular survival, and the growth rate at 40°C was similar to that at 37°C [14, 15].

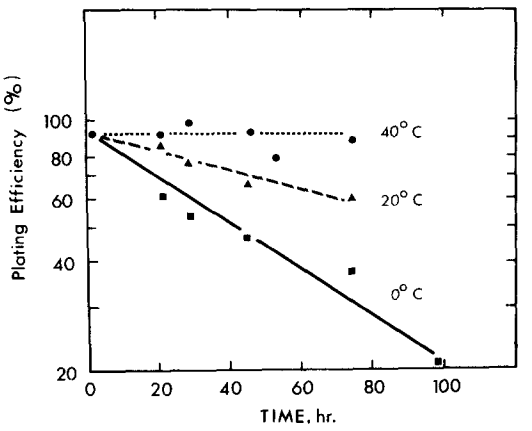

Fig. 1. Plating efficiency as a function of time at 0–4 (■), 20° (▲) or 40°C(●). Cells were incubated at the specified temperature for the indicated time, returned at 37°C and assayed for colony formation. These data are from a single representative experiment.

The incubation of cells at 40°C for 7 hr immediately prior to exposure to 10 or 20 min at 45°C induced a small degree of heat resistance enhancing cell survival by a factor of 1.6 by 2 hr [8, 16]. However, if 45°C-hyperthermia (10 or 20 min) was followed by incubation at 40°C for up to 6.5 hr, survival was reduced at an exponential rate of −0.33 per hr (Fig. 2) [8, 16].

Post-incubation at 0°–4° or 20°C for 2 hr after 45°C-hyperthermia enhanced cell survival by a factor of 1.7 (Fig. 2). Further incubation at 20°C had no additional effect on cell survival, whereas, additional exposure to 0°–4°C reduced survival back to 37°C control levels by 5 hr after the heat treatment (Fig.2). In contrast, incubation at 0°–4° or 20°C for up to 7 hr immediately prior to 45°C-hyperthermia or irradiation had no significant effect on either response (data not shown).

The enhanced survival with post-hyperthermia incubation at suboptimal tem-

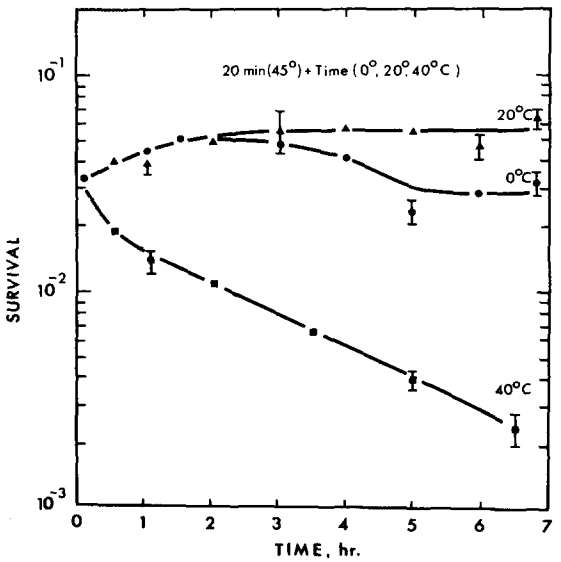

Fig. 2. Cellular surviving fraction as a function of the time of incubation at 0°–4° (●), 20° (▲) *or* 40°C (■) *following* 20 min *at* 45°C *and before returning to* 37°C.

peratures is similar to that occuring after post-irradiation incubation at 20°C and constitutes the operational definition of repair of potentially lethal radiation damage [6, 7]. Therefore, the recovery demonstrated in Fig. 2 may be defined, by analogy, as the repair of potentially lethal hyperthermia damage.

The accumulation of hyperthermic sublethal and potentially lethal damage (H-SLD and H-PLD, respectively) was studied by comparing the effect of a single heat treatment of 20 min at 45°C with that of two fractionated treatments, each of 10 min at 45°C, with intervening incubation periods at 0°–4°, 20°, 37° or 40°C (Fig. 3). With fractionation the total time at 45°C is slightly less than that with a single combined treatment due to the second temperature transient. An upper limit for this artifact can be established by comparing survival values for 0 and 30 min fractionation intervals (Fig. 3). This upper limit is 14%.

Incubation at 37°C for up to 7 hr between 10 min heat fractions enhanced cell survival by a factor of about 5.8 (Fig.3, Table 1). The recovery data fit a straight line, however, the visual fit was chosen so as not to suggest an artificially increased recovery ratio. This enhanced survival reflects not only the repair of H-SLD but to some extent the induction of thermotolerance although thermotolerance development occurs between 4 and 7 hr post-45°C-hyperthermia [16].

Analysis of the D_0 of a subsequent 45°C-hyperthermia survival curve determined 7 hr after an initial heat treatment of 10 min at

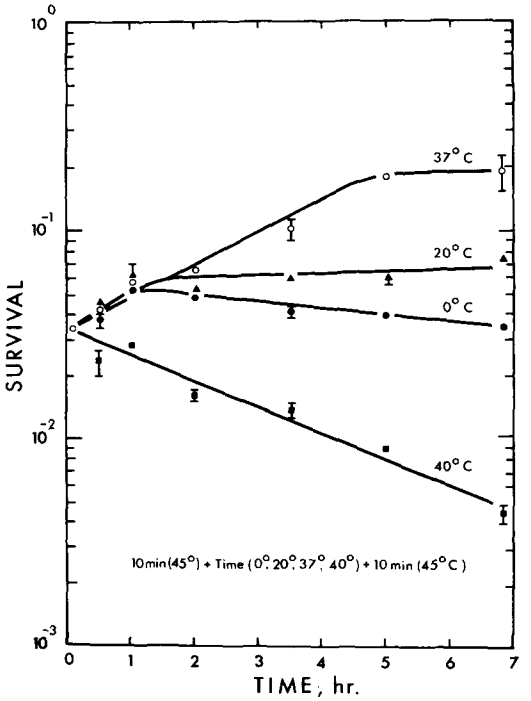

Fig. 3. Cellular surviving fraction as a function of interfraction interval separating two 10 min *at* 45°C *treatments. During the fractionation interval cells were maintained at* 0°–4° (●), 20° (▲), 37° (○) *or* 40°C (■). *Survival following* 10 min *at* 45°C = 3.0×10^{-1}.

45°C shows that the induction of thermotolerance was inhibited by 80, 68 and 12% during the 7 hr incubation at 0°–4°, 20° and 40°C, respectively (Table 2). Incubating cells at 0°–4°C post-45°C-hyperthermia resulted in a similar survival as maintaining the temperature at 0°–4°C between divided heat treatments. This suggests that both repair of H-SLD and the induction of thermotolerance are inhibited at 0°–4°C. At 20°C the induction of thermotolerance was inhibited by approximately 70% (Table 2), and comparison of Figs. 2 and 3 also suggests that the repair of H-SLD was reduced at 20°C (Table 1).

Incubation at 40°C reduced cell survival exponentially with time at a similar rate either when following a single treatment of 20 min at 45°C or between two treatments of 10 min at 45°C each (Figs. 2 and 3). Although the immediate effects ($\leqq$0.5 hr) were somewhat different such that the sequence 20 min at 45°C→30 min at 40°C was more toxic than 10 min at 45°C→30 min at 40°C→10 min at 45°C, the result implies that neither H-SLD nor H-PLD was repaired during during 40°C incubation between 45°C heat treatments. Instead, since the decrement in survival with incubation during the fractionation interval at 40°C was similar to the increased survival

Table 1. Modification of 45°C-hyperthermia/radiation induced cell killing by alteration of incubation tempaerature

Treatment sequence*	Survival ratio (S_T/S_0)† after 6.5 hr incubation at temperature T°		
	20°C	37°C	40°C
T° →H	1.0	1.0	1.6‡
H →T°	1.7	1.0	0.07
T° →X	1.0	1.0	0.7‡
X →T°	1.2	1.0	0.4‡
H →T°→H	2.0	5.8	0.2
HX→T°	2.0	1.0	0.02
H →T°→X	1.5	1.2	0.06
X →T°→H	1.6	1.6	3.0

*T° = Temperature of 6.5 hr incubation period.
H = Hyperthermia of 10 min at 45°C.
X = Irradiation of 400 rad.

†S_T = Cellular surviving fraction after treatment sequence with 6.5 hr incubation at temperature T°.
S_0 = Cellular surviving fraction after single treatment at 37°C.
‡Data taken from Ref. 8.

Table 2. Effect of temperature on the induction of thermotolerance

	Single dose control	10 min at 45°C→7 hr at 0, 20, 37 and 40°C→t* at 45°C→37°C			
		0–4°C	20°C	37°C	40°C
D_0 of 45°C. hyperthermia survival curve	3.6† ±0.1‡	4.5±0.2	7.0±0.4	21.3±0.7	18.7±0.6
Thermotolerance ratio§	1	1.2	1.9	5.9	5.2
Inhibition of thermotolerance (%)	—	80	68	0	12

*t = Time in min at 45°C.
†D_0 in min at 45°C.
‡1 S.E.M.
§Ratio of D_0 after thermotolerance induction to D_0 of single dose hyperthermia survival curve.

when the fractionation interval was at 37°C (0.2 vs 5.8, respectively, see Table 1) H-SLD may, in fact, at 40°C be converted to lethal damage [8, 16].

Radiation

Post-irradiation incubation for 4 hr at 20°C after a dose of 400 rad, but not at 0°–4°C, enhanced survival by a factor of 1.2 (Fig.4, Panel B; Table 1). For incubation at 0°–4°C or 20°C in excess of 4 hr, either before or after irradiation, the plating efficiency decreased due to hypothermic damage (Fig. 1). The repair of X-PLD in CHO cells at 20°C (Fig. 4B) is not statistically significant but is in agreement with results reported by others [6, 7].

In contrast to the effects at 20°C, pre-incubation at 40°C before irradiation with 400 rad reduced cell survival exponentially at a rate of 0.04/hour, while post-incubation at 40°C reduced survival within the first hour after 400 rad by a factor of 0.2 after which survival declined exponentially at the same rate as pre-incubation at 40°C (data in Reference 8 and cited here for comparison.)

45°C-Hyperthermia–radiation interaction

The interaction of H-PLD and X-PLD was studied by comparing the cell survival following 45°C-hyperthermia only, or irradiation only, combined with 0°–4°, 20° or 40°C incubation, respectively, to the cell survival following combinations of 45°C-hyperthermia

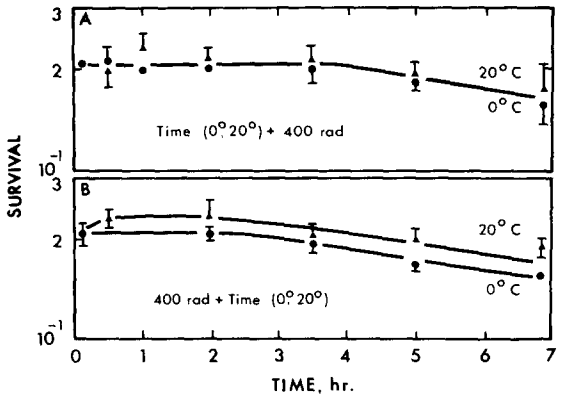

Fig. 4. Panel A: cellular surviving fraction as a function of the time of incubation at 0°–4° (●) or 20°C (▲) before irradiation with 400 rad and transfer to 37°C. Panel B: cellular surviving fraction as a function of the time of incubation at 0°–4° (●) or 20°C (▲) following irradiation with 400 rad and before transfer to 37°C.

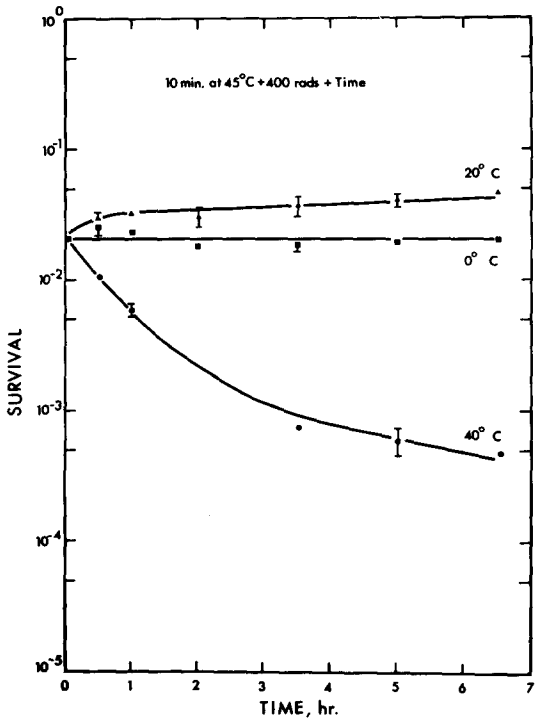

Fig. 5. Cellular surviving fraction as a function of the time of incubation at 0°–4° (■), 20° (▲) or 40°C (●) following 45°C-hyperthermia and irradiation. Cells were heated for 10 min at 45°C, immediately irradiated with 400 rad (3 min) and incubated at the designated temperature for the indicated time before transfer to 37°C. Survival following 10 min at 45°C = 3.0×10^{-1}; 400 rad = 2.0×10^{-1}.

and radiation with post-treatment incubation or interfraction incubation at 0°–4°, 20° or 40°C.

Both X-PLD and H-PLD were repaired during the first 2 hr at 20°C following either treatment alone (Fig. 2 and 4); the magnitude of each was 1.2 and 1.7, respectively (Table 1). Recovery after combined hyperthermia and irradiation followed by incubation at 20°C was also complete by 2 hr, and the combined repair of X-PLD and H-PLD enhanced cell survival by a factor of 2.0 after a 6.5 hr post-incubation period (Table 1). This suggests independence of X-PLD and H-PLD. Similarly, a 0°–4°C post-treatment incubation temperature which resulted in no X-PLD repair and only transient H-PLD repair left survival unchanged after combined hyperthermia and irradiation (except for the transient H-PLD repair).

A 6.5-hr incubation period at 40°C after either 45°C-hyperthermia or irradiation reduced survival by a factor of 0.07 or 0.4, respectively (Table 1), so that one could expect a 0.03 reduction in survival after a combined treatment, assuming an independent interaction. An experimentally determined factor of 0.02 (Fig. 5, Table 1) supports to a first approximation the hypothesis of the independent interaction of H-PLD and X-PLD at 40°C.

To further test the interaction of 45°C-hyperthermia and radiation damage fractions of 400 rad and heat treatments of 10 min at 45°C were separated and the cells incubated at either 0°–4°, 20°, 37° or 40°C between fractions (Figs. 6 and 7). The results of these fractionation experiments were similar to those obtained when hyperthermia and radiation were given conjointly. The difference between the treatment sequences HX+t at 0°, 20° and 40°C, and H+t at 0°, 20° and 40°C+X lies in the exchange of post-irradiation incubation for pre-irradiation incubation.

The results in Fig. 4 indicate that this exchange should reduce the cell survival component for irradiation from that in Fig. 5 by a factor of 0.8 when the interaction interval was 20°C, a factor of 1.0 for 0°–4°C, but enhance survival by 1.75 for 40°C incubation. Figure six verifies the expected survival values for the 0°–4° and 20°C interfraction temperatures. At 40°C, however, survival was increased by a factor of about 3.0 over the expected result of 1.75 for a 6.5-hr fractionation interval. However, the independent survival for H+t at 40°C of 0.07, and t at 40°C+X of 0.7, would yield a S_t/S_0 of 0.05 which is quite close to the observed survival ratio of 0.06 (Table 1).

The results of the sequence X+t at 0°, 20°, 37° and 40°C+H are shown in Fig. 7. From the above hypothesis of independent interaction one would predict that the exchange of 20°C post-incubation after 45°C-hyperthermia

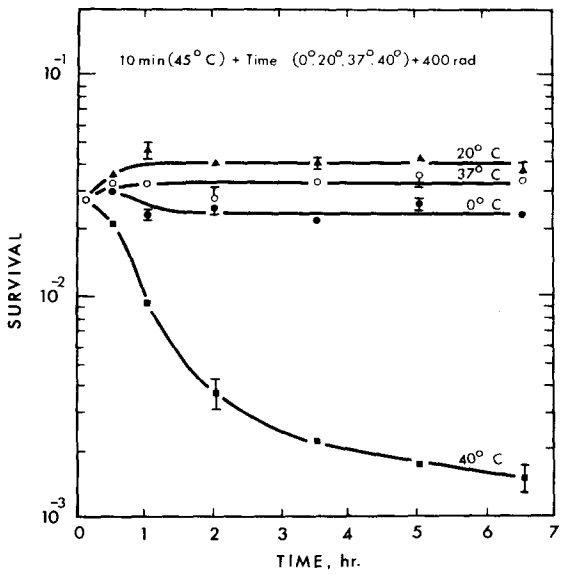

Fig. 6. Cellular surviving fraction as a function of the time of incubation at 0°–4° (●), 20° (▲), 37° (○) or 40°C (■) between 45°C-hyperthermia and irradiation. Cells were heated for 10 min at 45°C, incubated at the designated temperature for the indicated time, transferred to 37°C, and immediately irradiated with 400 rad. Survival following 10 min at 45°C = 3.0×10^{-1}; 400 rad = 2.0×10^{-1}.

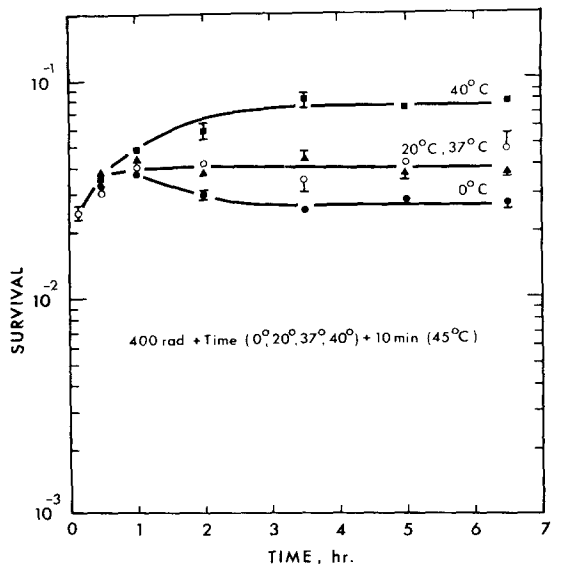

Fig. 7. Cellular surviving fraction as a function of the time of incubation at 0°–4° (●), 20° (▲), 37° (○) or 40°C (■) between irradiation and 45°C-hyperthermia. Cells were irradiated with 400 rad, incubated at the designated temperature for the indicated time, and heated for 10 min at 45°C before transfer to 37°C. Survival following 400 rad = 2.0×10^{-1}; 10 min at 45°C = 3.0×10^{-1}.

for an equal pre-incubation would reduce the survival observed in Fig. 5 by about 0.6 since in the latter case H-PLD would not be repaired. Similarly, survival should be unchanged when cells are incubated at 0°–4°C for a period greater than 3 hr either before or after 45°C-hyperthermia. The results in Fig. 7 show survival was reduced by a factor of 0.8 over the results shown in Fig. 5 when the sequence $X \rightarrow t_{20} \rightarrow H$ replaced $HX \rightarrow t_{20}$. The results for the 0°–4°C incubation were unchanged by sequencing as would be expected.

With 40°C incubation a similar exchange of pre- for post-incubation should avoid the toxic effects of the 45°→40°C sequence, and instead induce a small degree of heat resistance (a factor of 1.6 above 37°C control, see Table 1). Such expected results are verified in Fig. 7. Instead of the decrement in survival of 0.02 for $HX \rightarrow t_{40}$, or 0.06 for $H \rightarrow t_{40} \rightarrow X$, survival was increased to $S_t/S_0 = 3.0$ for the sequence X + 6.5 hr at 40°C + H (Table 1).

It is interesting to note that the survival values for the interfraction temperatures of 20° and 37°C for the sequence $X \rightarrow t \rightarrow H$ were similar. Since neither incubation at 20° nor 37°C before 45°C-hyperthermia affected cell survival, this result implies that the repair of X-PLD which occurs at 20°C is comparable in magnitude to the repair of X-SLD at 37°C, and after 3 hr neither remains to interact with heat damage.

DISCUSSION

Evidence strongly suggests that hyperthermia and radiation affect distinct cellular targets. Differences in the cell cycle dependence of killing by hyperthermia or irradiation [17, 18], the correlation of chromosome aberrations with cell lethality [6, 19], BUdR sensitization [19, 20], DNA strand break induction and repair [21, 22], oxygen sensitization [23, 24], inactivation energies for cell killing [4, 17, 25], the effect of radioprotective sulfhydryl agents [26] and thermotolerance [13, 16, 27] constitute such evidence. The question remains, however, to what extent do sublethal or potentially lethal hyperthermia lesions interact with sublethal or potentially lethal radiation lesions? The incubation of asynchronous CHO cells at either 0°–4°, 20° or 40°C for up to 7 hr is a relatively nonlethal treatment and most cells recover readily upon return to 37°C. Utilizing these temperatures to modify repair processes, it was noted that interaction of sublethal or potentially lethal hyperthermia damage and radiation damage was independent.

Hyperthermia treatments in the range of 40°–43°C primarily affect the cellular radiation response by inhibiting repair of SLD (modification of shoulder) [4, 9, 10] whereas higher temperatures in the range of 43°–46°C

primarily sensitize cells to lethal or potentially lethal damage (modification of slope) [4, 13, 19]. When 40° and 45°C-hyperthermia are combined with irradiation both inhibition of repair and radiosensitization occur [8, 9]. Post-heating, but not pre-heating, at 43°C inhibits repair of X-PLD in plateau phase Chinese hamster HA-1 cells [28], incubation of heated plateau phase cells in buffer inhibits repair of H-PLD [28], and incubation of irradiated or heated exponentially growing CHO cells at 20°C permits the repair both of X-PLD and H-PLD [6, 7, Figs. 3, 4]. Interestingly, hyperthermia pre-irradiation, but not post-irradiation, both sensitizes cells in plateau phase to killing [28] and inhibits the rejoining of radiation-induced single and double-strand breaks in DNA [21, 22], but does not inhibit repair of X-PLD.

The results presented herein indicate that the repair of H-PLD occurs transiently at 0°–4°C and permanently at 20°C, but not at 40°C. Repair of H-SLD is inhibited at 0°–4°, 20° or 40°C. Furhtermore, H-SLD may be converted to lethal damage at 40°C since S_t/S_0 was 5.8 when the interfraction temperature was 37°C but was 0.2 when the interfraction interval was kept at 40°C (Fig. 3, Table 1, also Refs. 8, 16). The interaction of sublethal and potentially lethal radiation damage with sublethal and potentially lethal 45°C-hyperthermia damage at incubation temperatures of 0°–4°, 20° or 40°C could best be understood as a super-position of the separate effects of either modality alone combined with incubation at 0°–4°, 20° or 40°C.

The repair of X-PLD at 20°C in exponentially growing CHO cells leads to approximately a 2-fold increase in survival and is a function of shoulder width ([6, 7] Fig. 4). However, plateau phase Chinese hamster HA-1 cells repair X-PLD during post-irradiation incubation at 37°C leading to approximately a 10-fold increase in survival [28] which is primarily a function of the slope of the radiation survival curve [11]. Therefore, the interaction of X-PLD and H-PLD observed by Li *et al.* in the plateau phase HA-1 cells [28] and the lack of interaction observed in exponentially growing CHO cells under subphysiological temperatures (Figs. 5–7) may be a function of lesion expression in different culture conditions. Although hyperthermia unquestionably affects the expression of radiation damage, it most likely does so by a mechanism distinct from hyperthermia-induced cell killing.

The only results which significantly deviate from the model of additivity were those at 40°C for the sequence $X \rightarrow T_{40} \rightarrow H$; a S_t/S_0 ratio of 0.6 was expected and 3.0 was observed. Other sequences of post-45°C-hyperhtermia or post-irradiation incubation at 40°C, which by itself is non-lethal, greatly enhanced lethal response. It is as if this temperature not only inhibited the repair of potentially lethal lesions, but led to the conversion of sublethal lesions to lethal events.

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