Interactions between Hyperthermia and Irradiation in Two Human Lymphoblastic Leukemia Cell Lines *\textit{in Vitro}*

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**ABSTRACT**

Thermal radiosensitization was studied in two human T-cell acute lymphoblastic leukemia cell lines (JM and MOLT3) with regard to heat-irradiation sequence and heating duration.

In MOLT3 thermal radiosensitization was maximal when 43.5°C hyperthermia immediately preceded or followed irradiation; at 41.5°C, radiosensitization was maximal with hyperthermia immediately before or up to 3 h after irradiation. In JM, enhancement of radiation killing was unexpectedly maximal when 41.5 or 43.5°C hyperthermia preceded irradiation by 2 to 4 h.

Thermal radiosensitization increased exponentially with increasing duration of heating at 41.5°C for at least 3 h in MOLT3. In contrast, in JM, radiosensitization increased exponentially for 1.6 h but additional heating (up to 3 h net heating) had no appreciable further effect on radiation killing.

For JM, repair of single and double stranded DNA breaks was investigated using alkaline and neutral elution techniques to determine whether the unusual results regarding heat-irradiation sequencing were related to effects of heat on repair of DNA damage. These studies were unable to detect significant differences in repair of single or double stranded DNA breaks between unheated control cells and cells heated at 41.5°C for 1 h ending 4 h before irradiation. The direct cytotoxicity of hyperthermia was also studied in both cell lines.

**INTRODUCTION**

Thermal radiosensitization, the ability of heat to increase the sensitivity of cells to ionizing irradiation, has been extensively investigated because of its potential clinical application (1, 2). Much of this effort has been directed at determining how the development of thermal radiosensitization depends on the sequencing of heat and irradiation (3–9), net heat dose, and thermotolerance (10, 11), a temporary resistance to thermal injury induced by prior heat exposure.

These studies have shown several characteristic patterns of heat-irradiation interactions which apply to many mammalian cells. First, radiosensitization has been found to be maximal when heat and irradiation are given simultaneously rather than sequentially (1, 3, 5–9). As a corollary to this, in cases in which truly simultaneous heating and irradiation were not studied, radiosensitization invariably decreased as the interval between heat and irradiation increased (4). Second, thermal radiosensitization increases as the degree of thermal injury increases (2, 12). Finally, thermotolerance may decrease the magnitude of thermal radiosensitization (2, 10–12).

The clinical utility of combining irradiation and local hyperthermia is now generally recognized. Recently, considerable progress has been made toward combining ionizing irradiation and systemic hyperthermia for the treatment of human malignancies (13–15). In the specific setting of systemic hyperthermia (but equally relevant to local and regional hyperthermia) it would be valuable to know whether the principles outlined above apply to human malignancies at temperatures at or below 41.8°C which are clinically practical using existing whole body hyperthermia technology (16). It happens, however, that few studies have considered heat-irradiation interactions in human cells in this temperature range (7, 12, 17); most data have concerned hyperthermia at temperatures well above 42°C (4, 5, 9, 10, 12, 17–21). Studies at temperatures above 41.8°C have suggested that hyperthermic radiosensitization is related to the development of thermal injury (2, 12). However, there is ample evidence to indicate that mechanisms of thermal injury may differ at temperatures above and below about 42–43°C (3, 7, 22–24). Consequently, it is conceivable that thermobiology principles derived from observations at temperatures above 42°C may not apply to the temperature range of combined systemic hyperthermia and radiotherapy.

In order to investigate this possibility, we studied the interactions of heat and irradiation *\textit{in vitro}* at 41.5 and 43.5°C with regard to heat-irradiation sequencing and net heat dose in two human, T-cell acute lymphoblastic leukemia cell lines, JM and MOLT3 (25).

**MATERIALS AND METHODS**

Cell Lines. JM and MOLT3 were kindly provided by Dr. S. Z. Salahuddin of the National Cancer Institute. Clones of JM and MOLT3 arising in media solidified with 0.3% Difco Bacto-Agar (Difco Laboratories, Detroit, MI) were isolated, grown in suspension in antibiotic supplemented RPMI 1640 (GIBCO Laboratories, Grand Island, NY), frozen, and replenished from frozen stock as we describe elsewhere (26). During exponential growth in suspension cultures, these clones of JM and MOLT3 had cell density doubling times of 26 and 29 h, respectively.

Survival Assays. Survival was assessed by counting clones arising in soft agar plated in 6-cm diameter disposable Petri dishes. Cells were suspended in RPMI 1640 containing 20% fetal bovine serum, 0.3% agar, 100 units/ml penicillin, and 100 µg/ml streptomycin and layered on top of 5 ml of RPMI 1640 solidified with 0.5% agar and containing 10% fetal bovine serum (26). Pipetting several times gave at least a 99.9% single cell suspension with both cell lines. After incubation for 14 to 18 days (JM) or 18 to 22 days (MOLT3) macroscopic colonies at least 0.1 mm in diameter were counted using 2-fold magnification. The cloning efficiency of untreated JM and MOLT3 cells averaged 38 ± 5.0% (SE) and 42 ± 4.3%, respectively.

Hyperthermia Methodology. During experiments exponentially growing, mycoplasma-free cells (Mycotrim-TC, New England Nuclear Research Products, Boston, MA) were seeded in disposable 15-ml centrifuge tubes in RPMI 1640 containing a dual bicarbonate and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer system and heated by placing tubes in a water bath with continuous shaking. This permitted stable and uniform gas mixtures (5% CO₂-95% air), control of pH to 7.4 ± 0.05, and control of temperature to within 0.05°C during treatments (26). Cells used in alkaline and neutral elution studies were grown and labeled in 25-cm² tissue culture flasks (Becton-Dickinson, Oxnard, CA). Cells were heated by suspending the flasks in 41.5°C water baths for 1
Cells were centrifuged and resuspended in 3 ml PBS, and 3 x 10^6 cells/ml in 25-cm² tissue culture flasks) and maintained at room temperature with 250 kVp X-rays at 1.78 Gy/min (3 ml at 1 x 10^6 cells/ml in 25-cm² tissue culture flasks) and maintained at room temperature for various intervals after irradiation. At the appropriate time after irradiation, 40 ml of ice-cold PBS* were added to each sample. Cells were centrifuged and resuspended in 3 ml PBS, and 3 x 10^6 H labeled reference cells (which had been irradiated to 3.0 Gy on ice in phosphate buffered saline) were added. Cell samples were then lysed on 0.8-µm polycarbonate filters with 1% sodium dodecyl sulfate-0.02 M EDTA (pH 12.1) containing 0.02 M EDTA and 0.1% sodium dodecyl sulfate. For each sample, six fractions were collected at hourly intervals.

Repair of Single Stranded DNA Breaks. The kinetics of repair of DNA single strand breaks in irradiated JM cells was determined by alkaline elution. Cells were labeled with [3H]thymidine at 0.02 µCi/ml for 48 h and for an additional 24 h in medium free of [3H]thymidine to establish the label into high molecular weight DNA. Reference cells were similarly labeled with [3H]thymidine. 14C-labeled cells in RPMI 1640 plus 20% fetal bovine serum were irradiated to 4.0 Gy at room temperature with 250 kVp X-rays at 1.78 Gy/min (3 ml at 1 x 10^6 cells/ml in 25-cm² tissue culture flasks) and maintained at room temperature for various intervals after irradiation. At the appropriate time after irradiation, 40 ml of ice-cold PBS* were added to each sample. Cells were centrifuged and resuspended in 3 ml PBS, and 3 x 10^6 H labeled reference cells (which had been irradiated to 3.0 Gy on ice in phosphate buffered saline) were added. Cell samples were then lysed on 0.8-µm polycarbonate filters with 1% sodium dodecyl sulfate-0.02 M EDTA (pH 10). DNA was eluted from the filters with 2% tetra-propylammonium hydroxide (pH 12.1) containing 0.02 M EDTA and 0.1% sodium dodecyl sulfate. For each sample, six fractions were collected at hourly intervals.

Repair of Double Stranded DNA Breaks. Repair kinetics of DNA double strand breaks at room temperature was done using neutral elution (pH 7.1). Cells were labeled as for alkaline elution. Cells in RPMI 1640 plus 20% fetal bovine serum were irradiated to 100 Gy on ice in a 137Cs irradiator at a rate of 8.9 Gy/min. After irradiation, cells were centrifuged, resuspended in room temperature medium, and then maintained at room temperature. At appropriate times after irradiation, aliquots (3 x 10^6 cells) were diluted in ice-cold PBS and concentrated, and H labeled reference cells were added. Elution was done as for alkaline elution except that all solutions including lysis solution were adjusted to pH 7.1 and proteinase K was used in the lysis solution.

Ionizing Irradiation. Survival experiments used two 137Cs sources yielding 1.27 and 7.89 Gy/min, respectively. Differences in dose rate had no observable effect on the results obtained.

In Figs. 1 to 6, bars indicate the SEM. Bars which are so small as to be encompassed in the accompanying symbol are not shown. Experiments were repeated at least twice and were performed at least in duplicate.

RESULTS

Fig. 1 shows the hyperthermia sensitivities of JM and MOLT3. MOLT3 is more sensitive to hyperthermia than is JM at both 41.5 and 42.5°C but JM and MOLT3 have qualitatively similar survival curves. In both cell lines survival is not reduced by 3 h at 40.5°C. At 41.5°C survival initially decreases but then plateaus as the duration of heating increases. In both cell lines at 42.5°C, survival decreases exponentially as the duration of hyperthermia increases for at least 3 h of heating. The effect of heating duration on radiosensitivity is shown in Fig. 3. In both cell lines, when 41.5°C hyperthermia immediately precedes a given dose of irradiation, radiosensitivity changes little for the first 20 min of heating. Thereafter, the survival of JM decreases exponentially until a net 1.6 h of heating. After 1.6 h, the slope changes abruptly and additional preirradiation heating does not further decrease cell survival. The same effect is apparent when survival is corrected for the effects of direct heat killing. In contrast, for MOLT3, after the first 20 min of heating, survival decreases exponentially with increasing duration of heating for at least 3 h with or without correcting for direct heat toxicity.

Fig. 4 describes the survival of JM cells as a function of heat-irradiation sequence. Data for heated, irradiated cells has been corrected for direct heat killing and then normalized to that of irradiated but unheated control cells (100% survival). This format facilitates comparisons between trials at different temperatures and in different cell lines.

In JM, maximal thermal enhancement occurs when a 1-h 41.5°C heat exposure ends 2 to 4 h before irradiation (4.0 Gy). Thermal radiosensitization is apparent even when 41.5°C hyperthermia ends 6 h prior to irradiation.

Fig. 4 also shows the effects of heat-irradiation sequence on thermal radiosensitization when 4.0 Gy are given with 43.5°C.

Footnote:

* The abbreviation used is: PBS, phosphate buffered saline.
THERMAL RADIOSENSITIZATION

Fig. 3. Thermal radiosensitization as a function of duration of preirradiation hyperthermia in JM and MOLT3. Cells were heated at 41.5°C for varying lengths of time immediately before receiving 4.0 Gy (JM) or 1.0 Gy (MOLT3). Including time at 37.0°C, all cell samples were incubated for 3 h. Survival was calculated as a percentage of the survival of irradiated, unheated controls with and without correcting for direct heat toxicity. Bars, SE.

Fig. 4. Heat-irradiation sequence and survival of JM. Cells were heated for 1 h at 41.5°C or for 20 min at 43.5°C at varying times before (negative time values) and after (positive time values) irradiation to 4.0 Gy. Points indicate midpoints of heat exposures. Bars, SE. Survival was corrected for direct heat killing and expressed as a percentage of the survival of unheated, irradiated controls. Less than 100% survival indicates thermal radiosensitization.

for 20 min. As occurs at 41.5°C, thermal radiosensitization is maximal in JM when 43.5°C heating ends well before irradiation; radiosensitization remains substantial even if 43.5°C hyperthermia precedes irradiation by as much as 9 h.

In the experiments shown, 41.5 and 43.5°C hyperthermia cause modestly different degrees of direct heat killing. The survival of JM after 41.5°C for 1 h (with no irradiation) is 67.9% compared to the survival of unheated, unirradiated controls. Survival is 33.1% after 43.5°C for 20 min. After correcting for direct heat killing, thermal radiosensitization is greater using 43.5°C for 20 min than using 41.5°C 1 h.

Fig. 5 describes the effects of heat-irradiation sequence on thermal radiosensitization in MOLT3 cells. Unlike in JM, maximal thermal enhancement occurs in MOLT3 when 41.5°C for 1 h immediately precedes irradiation (2.0 Gy) or follows irradiation by less than 3 h. Substantial radiosensitization occurs even when heating begins as much as 6 h after irradiation. Enhancement of radiosensitivity is less pronounced when 41.5°C precedes irradiation by 2 to 9 h.

In MOLT3, thermal radiosensitization at 43.5°C is much greater than at 41.5°C and remains substantial when heat is given well before or after irradiation. Again, 41.5 and 43.5°C hyperthermia cause modestly different degrees of direct heat killing. In the experiments shown (Fig. 5), survival after 41.5°C for 1 h is 68.3% compared to 41.0% after 43.5°C for 20 min.

As Fig. 5 also shows, thermal radiosensitization in MOLT3 peaks sharply as 43.5°C hyperthermia and irradiation are given closer together. However, at 41.5°C one can only roughly estimate the timing of heat and irradiation which gives maximal thermal radiosensitization (i.e., between about —1.0 and +4.0 h in Fig. 5). This behavior of MOLT3 contrasts with that of JM. In Fig. 4 the 41.5 and 43.5°C curves are qualitatively more similar.

In JM, the rates of repair of single and double stranded DNA breaks with or without preirradiation heating (41.5°C for 1 h ending 4 h before irradiation) were not significantly different as determined by least squares analysis (Fig. 6).

DISCUSSION

This study represents the only example, of which we are aware, in which heat-irradiation interactions are compared under identical experimental conditions in two cell lines derived from the same species and cell type. MOLT3 is quantitatively more sensitive to hyperthermia (Fig. 1) and to irradiation (Fig. 2) as single agents than is JM. Yet, JM and MOLT3 share certain features. These cell lines have qualitatively similar patterns of survival following hyperthermia alone (Fig. 1). For
both cell lines, the postirradiation survival curves lack a shoulder region either with or without preceding hyperthermia (Fig. 2), as is typical (7) of other human lymphoid neoplasms. A constant preirradiation heat exposure yields fairly equal thermal enhancement ratios in the two cell lines.

The molecular basis for thermal radiosensitization is not fully understood. Heat appears to enhance radiation killing by inhibiting enzymatic repair of lethal and sublethal radiation damage (17, 18, 20, 27). Repeated observations that radiosensitivity decreases as the interval between heat and irradiation increases are consistent with this notion (1–9, 20). These observations have encouraged theories to explain thermal radiosensitization which are based on mechanisms the effects of which are expected to peak with simultaneous hyperthermia and irradiation. In sharp contrast to these observations, JM cells uniquely show greatest thermal radiosensitization when 1 h of 41.5°C hyperthermia ends 2 to 4 h before irradiation (4.0 Gy) and less radiosensitivity when heat and irradiation are given closer together (Fig. 4).

For JM, qualitatively similar patterns of radiosensitization occur at both 41.5 and 43.5°C (Fig. 4). In contrast, for MOLT3, the curves representing relative survival using 41.5°C for 1 h and 43.5°C for 20 min do have some qualitative differences (Fig. 5). For both Figs. 4 and 5, the clearest difference between the 41.5 and 43.5°C curves is that thermal radiosensitization is greater and resolves more slowly at 41.5°C than it does at 43.5°C.

The origins of the behavior of JM are not known but could reflect effects of heat on the cell cycle distribution of JM cells such as redistributing cells to M or to late G2, which should increase (28) their radiosensitivity. This idea may be supported by studies (29) involving the CCRF-CEM cell line, another human T-cell acute lymphoblastic leukemia; 12 h after heating (42°C for 1 or 2 h) these cells accumulated in S and later in G2 and M. Parenthetically, however, studies (3, 4, 24) involving nonlymphoid malignancies have reported hyperthermia-induced cytokinetic effects different from those seen in CCRF-CEM.

It is unlikely that JM in Fig. 4 is simply expressing behavior typical for human lymphoid neoplasms at systemic hyperthermia temperatures since MOLT3 (Fig. 5) and MOLT4 (at 42°C; Ref. 7), two other human T-cell acute lymphoblastic leukemias (25), behave much differently. This difference may be due to predominance of different DNA repair mechanisms in these cell lines; alternatively, heat may unequally impair different repair mechanisms even in similar cell lines.

Fig. 6, which shows repair of irradiation injury in JM, does not clarify the possible molecular mechanisms of thermal radiosensitization in this cell line. Hyperthermia (41.5°C) ending 4 h before irradiation does not significantly slow repair of single or double stranded DNA in JM. These results are similar to data in other studies (30) of repair of single and double strand breaks which have not been able to detect thermal inhibition of DNA repair at systemic hyperthermia temperatures.

Existing studies (2, 10–12, 18) disagree on the importance of thermotolerance for efforts to combine hyperthermia and irradiation clinically. Generally in these studies a heat treatment, usually above 43°C, was used to induce thermotolerance and then radiosensitivity was assessed hours or days later. For clinical systemic hyperthermia it is more relevant to know the effects on radiosensitivity of thermotolerance which hypothetically may occur during hyperthermia at or below 41.8°C.

The experiments shown in Fig. 3 address this issue for JM and MOLT3. For both JM and MOLT3, thermal radiosensitization increases with increasing duration of preirradiation heating. However, for JM there is an abrupt breakpoint which correlates roughly with the expected onset (2) of thermotolerance. Thereafter radiosensitivity does not increase with additional heating. For MOLT3 such a breakpoint is not apparent. After correcting for direct heat killing, radiosensitivity increases steadily with longer preirradiation heating well beyond the point at which thermotolerance should (2) be present.

These data (Fig. 3) are consistent with two conclusions. First, for neither JM nor MOLT3 is there any hint of a decrease in the magnitude of thermal radiosensitization at a time when thermotolerance should be present. This idea is compatible with the finding (Fig. 4) that radiosensitization peaks in JM (Fig. 4) 2 to 4 h after heat injury at a time when thermotolerance should (2) be well developed (Fig. 1). Taken collectively, these observations imply that thermotolerance which might occur during systemic hyperthermia may not have a significant impact in clinical trials combining irradiation and systemic hyperthermia at temperatures near 41.5°C. This notion and supporting in vivo data are discussed elsewhere (31). A second conclusion is that the ideal duration of preirradiation hyperthermia varies between different cell lines. For JM the optimal duration of preirradiation hyperthermia might be about 1.6 h at 41.5°C. For MOLT3 longer heating may be preferable.

One experiment reported in the literature (21) is similar to those summarized in Fig. 3. In the murine BP-8 sarcoma, after about 1 h of 42.5°C preirradiation hyperthermia, a breakpoint occurs which is like that seen for JM (Fig. 3).

JM and MOLT3 are derived from the same cell type and species; qualitatively they have similar patterns of sensitivity to hyperthermia and to irradiation as single agents (Figs. 1 and 2). However, the data presented in Figs. 3 to 5 demonstrate that their responses to combined heat and irradiation can vary markedly. Furthermore, even in a given cell line (Fig. 5), the responses to heat and irradiation can vary qualitatively at different temperatures. Taken collectively these results suggest that caution should be exercised in making generalizations about heat-irradiation interactions.

REFERENCES

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