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Effect of Hyperthermia on the Survival of Normal Human Peripheral Blood Mononuclear Cells[1]

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ABSTRACT

Human peripheral blood mononuclear cells from normal healthy volunteers were exposed to elevated temperatures of 41–43° for up to 6 hr. Thereafter, the cells were stimulated with phytohemagglutinin *in vitro* in order to measure indirectly the surviving fraction. DNA replication in heated cells in response to phytohemagglutinin was found to be a sensitive indicator of thermal injury. Exposure to even 40° for 2 hr lowered thymidine incorporation at early time points after phytohemagglutinin stimulation, but the cells were able to recover from thermal injury after exposure for up to 4 hr at 42°. At 43°, exposure for even 1 to 2 hr caused irreversible damage. The changes in thymidine incorporation were not due to changes in endogenous nucleotide pools since parallel changes were observed in DNA polymerase activity. Thus, the heat sensitivity of normal human lymphocytes could be a limiting factor for use of hyperthermia as an adjunct to radiotherapy and chemotherapy of human cancer.

INTRODUCTION

The role of hyperthermia in the treatment of cancer, as an adjunct to radiotherapy and possibly chemotherapy, is increasingly recognized (5, 6, 13, 14, 20). Both local and systemic heat have been shown to be effective in the treatment of experimental and human tumors. However, the experience with human cancers has not been firmly established.

Studies on several established cell lines have shown that cell survival decreases above 42°. Arrhenius plots based on these survival curves indicate a break in the inactivation at 43° (16). In general, temperatures in excess of 43° are used for treatment of cancer. However, the safety of this temperature to normal tissues has not been adequately documented. In our earlier studies, we have observed that the upper limit of temperature that allows optimum response of human peripheral blood mononuclear cells to PHA[3] is 40° (3, 23). At 41° and 42°, DNA replication in PHA-stimulated lymphocytes is markedly reduced. Mononuclear cells are key cells in the immune response, and immunoregulation could play a significant role in the control of cancer. If hyperthermia is to be used widely in the treatment of cancer, it is important that the effects of exposure to elevated temperatures on the survival and proliferative response of lymphocytes be investigated. In this paper, we show that normal human mononuclear cells may recover from damage resultant from exposure to heat at 42° for up to 4 hr but not from the effect of exposure to 43° for as little as 1 to 2 hr. This heat sensitivity of normal human lymphocytes may be a limiting factor for the use of hyperthermia in the treatment of human cancer if temperatures in excess of 42° are used.

MATERIALS AND METHODS

Cells. Human peripheral blood was obtained from normal healthy volunteers. Mononuclear cells, including T-cells, non-T-cells, and monocytes (macrophages), were isolated by sedimenting the diluted blood on Ficoll-Hypaque gradients. The cells were washed 3 times with Roswell Park Memorial Institute Medium 1640, and the final cell concentration was adjusted to 1×10^6 cells/ml. Culture medium consisted of Roswell Park Memorial Institute Medium 1640 with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer supplemented with 1 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 20% fetal calf serum. One-ml cell aliquots in 10- x 125-mm Falcon plastic culture tubes were heated by incubating in a water bath at 40°, 41°, 42°, and 43° for varying lengths of time up to 6 hr. The cultures were then maintained in humidified CO_2 incubators at 37° for 1 hr prior to addition of PHA (PHA-M; Grand Island Biological Company, Grand Island, N. Y.). Fifty μl of recommended dilution of PHA were added to each culture, and the cultures were maintained at 37° for the indicated time.

Trypan Blue Dye Exclusion Test. The cell suspension (0.1 ml) was mixed with an equal volume of 0.4% trypan blue dye. The cells were left at room temperature for 5 min and then evaluated in both chambers of a hemocytometer. At least 200 cells were counted and, in case of discrepancy by more than 10% between the 2 chambers, the procedure was repeated. Cells were designated as viable if they excluded the dye and were not stained.

Measurement of [^{3}H]Thymidine Incorporation and DNA Polymerase Activity. Two hr prior to termination of cultures, [^{3}H]thymidine (2.5 μCi; 6.7 Ci/mmol) was added to each culture, and the cultures were returned to the incubator at 37°. At the end of 2 hr, the incorporation of thymidine was stopped by adding 5 ml of ice-cold 0.15 M KCl to each culture. The cells were pelleted at 2500 rpm × 10 min in an RC-2B refrigerated Sorvall centrifuge and were washed twice with ice-cold 0.15 M KCl. The cell pellet was then frozen and thawed, and the cell lysate was used for the assay of DNA polymerase. For the latter, [α-^{32}P]dTTP was used as one of the 4 deoxynucleotide triphosphate substrates, activated calf thymus DNA as added template, and Mg^{2+} as the metal activator. After incubation for 1 hr at 37°, radioactivity incorporated in an acid-insoluble product was quantitated. ^{3}H incorporation was taken as the rate of thymidine incorporated into DNA by PHA-stimulated lymphocytes in culture, and ^{32}P incorporation was taken as a measure of DNA polymerase activity *in vitro*. Details, control, and analysis of the product of the reaction have been described previously (2).

RESULTS

Measurement of Cell Viability by Trypan Blue Dye Exclusion Test. Human peripheral blood mononuclear cells were exposed

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[3] The abbreviation used is: PHA, phytohemagglutinin.

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to 41°, 42°, and 43° for different lengths of time. At the end of each exposure period, the viability of the cells was assessed by the dye exclusion method (Table 1). In addition, the total number and viability of the heat-treated cells were again determined after culturing for 12 hr at 37°.

Heating of normal lymphocytes at 41° or 42° for up to 6 hr and at 43° for up to 2 hr diminished trypan blue exclusion by less than 10%. At 43° for 3 hr, the number of stained cells declined by 25%. Reexamination by dye exclusion after subsequent incubation at 37° for 12 hr showed that a significant number of cells heated at 42° and 43° for 6 and 3 hr, respectively, were not able to exclude the dye (Table 1, legend). Thus, by the criteria of trypan blue dye exclusion, damage by heat may not be immediately apparent.

Response of Heated Mononuclear Cells to PHA. We have shown that response of X-irradiated lymphocytes to PHA provides an effective quantitation for the biological survival of irradiated lymphocytes (1). Likewise, we have measured the response of heated mononuclear cells to PHA to quantitate damage by heat. In this experiment (Chart 1), the mononuclear cells were first heated at 40°, 41°, 42°, and 43° for 2 hr and then stimulated with PHA. Thereafter, DNA polymerase activity and [^{3}H]thymidine incorporation were measured at 28, 46, and 70 hr after the addition of PHA. At 28 hr, the incorporation of thymidine in cells heated at all the temperatures was lower than in controls; the inhibition was proportionally greater in cultures heated at 43° compared to those heated at 40°. At subsequent intervals, the rate of thymidine incorporation in cultures heated at 40°, 41°, and 42° was similar to that of unheated controls, whereas cells exposed to 43° failed to recover. Similar results were obtained for the assay of DNA polymerase activity. The later measurement is independent of changes in transport or nucleotide pools in cells (2).

Effect of Heating for Various Lengths of Time at Different Temperatures. In order to delineate further the influence of temperature on responsiveness of lymphocytes to PHA, mononuclear cells were exposed to 40°, 41°, 42°, and 43° for varying lengths of time up to 6 hr. Thymidine incorporation and DNA polymerase activity were measured at 28, 46, and 70 hr after PHA (Chart 2). The *abscissa* defines the length of exposure of cells to elevated temperatures, and the *ordinate* gives the response of heated cells as a percentage of response of control cells. It is interesting to note that, at 28 hr, thymidine incorporation in cells heated at even 40° for 2 hr was impaired, even though DNA polymerase activity in these cultures was normal. This suggests that there are proteins more heat labile than polymerase that are required for thymidine incorporation. The effect of heat is most prominent at 28 hr. Apparently, the cells were able to recover from the damage caused by exposure to heat with the passage of time in culture at 37°. Thus, the incorporation of thymidine in cells heated at 43° for 1 hr was 5, 60, and 85% of the control at 28, 46, and 70 hr, respectively, after the addition of PHA. Likewise, the DNA polymerase activity increased from 42° to 65%. The recovery from exposing cells to 42° for up to 4 hr was almost complete by 70 hr in culture at 37°. On the other hand, exposure at 43° for 2 hr caused irreversible damage to mononuclear cells as measured by their inability to respond to PHA even when maintained in culture at 37° for 70 hr.

Table 1

Viability of cells measured by trypan blue dye exclusion test

Human peripheral blood mononuclear cells were cultured as described under "Materials and Methods." One-ml aliquots or 10^6 cells/ml in medium with 20% fetal calf serum were heated at 41–43° for different lengths of time. Immediately at the end of each heating period, the viability of the cells was determined by trypan blue dye exclusion method, as described under "Materials and Methods."

After incubation at 37°, 41°, and 42° for 6 hr and subsequently at 37° for 12 hr, the percentage of viable cells was 94, 89, and 65, respectively. After incubation at 43° for 3 hr followed by 12 hr at 37°, 25% of the cells were viable. At each time point, total cell counts were determined using a hemocytometer. There were no significant differences from the initial aliquot of 10^6/ml, indicating no loss of cells due to exposure to elevated temperature.

Incubation (hr)	% of viable cells after heating			
	37°	41°	42°	43°
0.5	NT[a]	NT	NT	96
1	98	99	98	92
2	98	97	98	90
3	97	96	97	75
4	97	96	97	NT
6	98	96	93	NT

[a] NT, not tested.

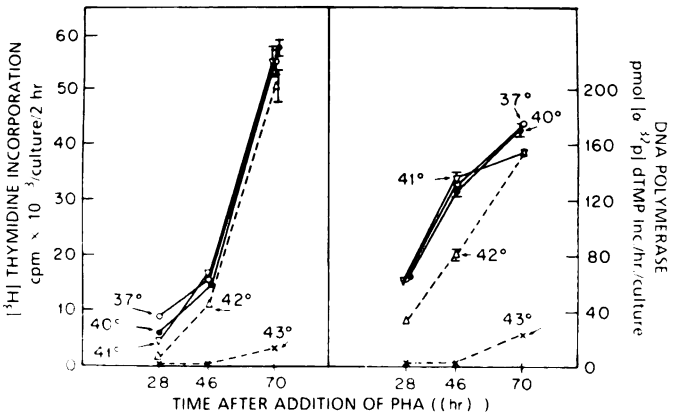

Chart 1. Human peripheral blood mononuclear cells were cultured as described under "Materials and Methods." One-ml aliquots containing 10^6 cells in medium with 20% fetal calf serum were heated at 37° for 2 hr. After a 1-hr incubation at 37°, PHA was added to the cultures. [^{3}H]Thymidine incorporation and DNA polymerase activity were measured as described under "Materials and Methods." ○, 37° (average, 5 cultures); ●, 40° (average, 3 cultures); ▽, 41° (average, 3 cultures); △, 42° (average, 3 cultures); and ×, 43° (average, 3 cultures). *Points*, mean; *bars*, S.D. The standard deviations at 28 and 46 hr were too small to be presented. Thymidine incorporation was significantly lower at 28 hr in cultures heated at 40°, 41°, 42°, and 43°, the values being 6089, 6069, 1278, and 23, respectively, compared to the 37° control of 8369. DNA polymerase activity was significantly lower only at 42° and 43°.

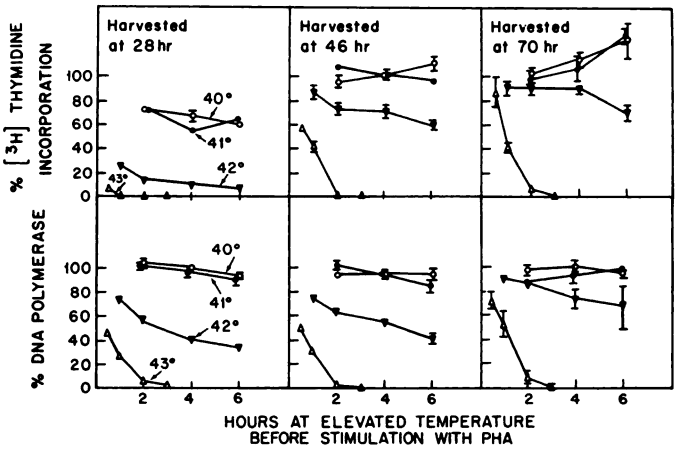

Chart 2. Human peripheral blood mononuclear cells were cultured as described under "Materials and Methods." The cells were heated at 40° to 43° for different lengths of time as indicated on *abscissa*. The response of heated cells to PHA is given as percentage of unheated control cells on the *ordinate*. *Points*, mean of 15 computations among 3 test cultures of the heated cells and 5 control cultures of unheated cells; *bars*, 1 S.D. ○, 40°; ●, 41°; ▲, 42°; and △, 43°.

DISCUSSION

A role for hyperthermia in the treatment of cancer is increasingly recognized. However, no guidelines exist for choosing an optimal schedule for heat therapy for the treatment of human cancers. Thermal sensitivity of normal human tissues is one of the major considerations in planning schedules for the treatment of cancer with heat. The results presented in this paper show that exposure of normal lymphocytes for up to 4 hr at 42° does not cause any permanent damage, while exposure at 43° for even 1 to 2 hr completely and irreversibly diminishes the proliferative potential of these cells. This indicates that the efficacy of heat treatment protocols, where temperatures in excess of 42° are used, should be reevaluated with respect to immune functions of lymphocytes in treated individuals. It also needs to be determined whether local heat treatment, without isolation of circulation, affects circulating lymphocytes.

Measurement of response to PHA after exposure of lymphocytes to noxious stimuli may provide a sensitive method for monitoring damage to these cells (1). We find that while 90% of the mononuclear cells exposed to 43° for 2 hr were capable of excluding trypan blue, they were not able to undergo DNA synthesis after stimulation with PHA. Although trypan blue dye exclusion test is a simple and rapid method for testing viability of cells (9, 11, 19), discrepancies between this test and other biological parameters of viability have been reported (13). The trypan blue dye test does not accurately reflect the thermal damage to lymphocytes (11, 15, 16, 21). Apparently the effect of heat on those cell membrane functions that determine dye exclusion is indirect or delayed. This is supported by the fact that, immediately after exposure to heat, only 25% of the cells heated at 43° for 3 hr were not able to exclude the trypan blue dye, while after additional incubation at 37° for 12 hr 75% of the heated cells were stained. A similar delayed effect of heat on cell membrane function has been reported by Lin *et al.* (17) and Schrek (22), though at higher temperatures.

The mechanisms by which elevated temperatures affect normal and malignant cells are unknown. The synergism between heat and radiation has been attributed to a heat-mediated inhibition of the repair of sublethal damage to DNA caused by radiation (4). Hyperthermia has been shown in most (8, 10) but not all studies (6, 20) to decrease the rate of both single- and double-stranded DNA repair (8). It is to be noted that eukaryotic DNA polymerase β, which has been hypothesized to function in DNA repair, is generically heat sensitive (12). Activity of DNA polymerase β is diminished *in vitro* by 50% after incubation for 15 min at 42°. It is conceivable that the potential synergism of heat and radiation may be mediated by selective inactivation of DNA polymerase β. This is in accord with recent studies on the correlations between inactivation of DNA polymerase β (when whole cells are heated) and radiosensitization and heat-induced cell killing (24).

Our preliminary studies on the effect of elevated temperature on DNA repair show that exposure of Somer cell line lymphoblastoid cells to 42° for 4 hr effectively inhibits the rejoining of X-ray induced single-strand breaks.[4] If these *in vitro* results can be extended and confirmed by clinical trials, exposure to 42° for 4 hr prior to X-irradiation may be a useful protocol for heat therapy as an adjunct to radiotherapy. The safety of 42° is supported by the observation that elevations of body temperature up to 41.8° under experimental conditions (7), and up to 41–42° with various natural infections and disorders of heat regulation, are well tolerated. Our *in vitro* studies reported here show that damage to normal human cells at 42° for 4 hr is reversible. We had chosen peripheral blood lymphocytes for these studies since lymphocytes are key cells for immune responses, and an intact immune system could be of considerable advantage in the overall control of cancer.

[4] Unpublished observations, S. S. Agarwal and L. A. Loeb.

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