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THE EFFECT OF HYPERTHERMIA ON THE DIFFERENTIATION OF LEUKEMIC CELL LINES

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ABSTRACT

Treatment of human promonocytic leukemic cell line U937 with mild hyperthermia in the temperature range of 40-43°C resulted in differentiation of these cells into monocyte/macrophage-like cells in a heat dose and time dependent manner. This process was accompanied by marked morphological, functional and proliferational changes. U937 cells which normally grow in supension in the logarithmic phase of growth showed marked inhibition in proliferation after treatment with heat in comparison with controls, without significant decrease in cell viability. The clonogenicity of these cells in semisolid agar cultures was also reduced upon heat treatment. Heat treatment increased the fraction of cells which could reduce nitro blue tetrazolium (NBT) and phagocytize latex particles. These data demonstrate that heat treatment can induce differentiation of U937 cells into monocytes/macrophages and thus have possible applications in treatment of leukemia. Temperatures higher than 43°C or exposures of longer than 30 minutes at such high temperatures resulted in cytotoxic effects.

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INTRODUCTION

It has been suggested that neoplasms are composed of malignant stem cells that have limited capacity for differentiation. This observation has provided the basis of the interesting possibility of treatment of various cancers and leukemia by induction of differentiation in malignant cells.[1]

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Development of human myeloid/leukemic cell lines has provided useful means for studying the regulation of cell proliferation and differentiation as useful models *in vitro*.[2] One such example is the human promonocytic cell line (U937) which was established by Christer Sundstrod and Kenneth Nilson in 1976.[3] U937 cells have a potential to differentiate into monocyte/macrophage-like cells under the influence of several factors including phorbol ester tetradecanoiyl phorbol acetate (TPA), phorbol myristate acetate (PMA),[4,5] dimethylsulfoxide (DMSO)[6], vitamin D_3 and it's metabolites,[7,8] formyl methionyl leucylphenylalanin (FMLP), chemotactic peptides,[9] gamma interferon[10] and myosin light chain kinase inhibitor.[11]

Monocytes and macrophages play a central role in the

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primary defensive response of the body by their ability to present foreign antigens to T-cells, to phagocytize microorganisms invading the body, and to mediate various defense mechanisms against neoplasia.[12,13] Many of these factors which induce differentiation in U937 cells are optimally active at concentrations which are cytotoxic or have many harmful side effects on normal cells.[14] Therefore exploring the factors which can induce differentiation in leukemic cells with fewer cytotoxic side effects and a higher rate of proliferation inhibition has been of primary importance and a subject of intensive investigation.

Hyperthermia (HT) or heating of cells at temperatures above 37°C results in toxicity to mammalian cells and can be directly lethal to malignant cells.[15] Prolonged exposure to mild HT or fractionated heating may lead to a heat resistance commonly known as thermotolerance.[16] HT has also been reported to radiosensitize certain cell types and result in enhanced cytotoxicity and cell death.[17]

In this work we have studied the effect of mild HT with limited cytotoxicity on the differentiation of U937 leukemic cell lines using various criteria for differentiation.

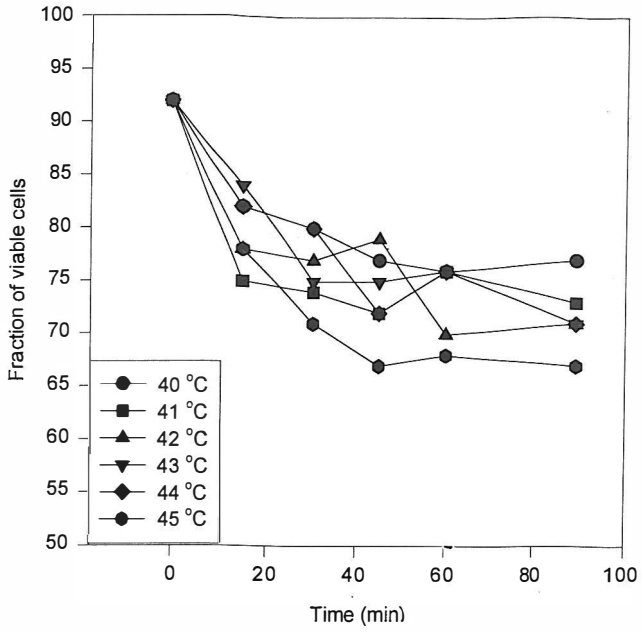

Fig. 1. Fraction of viable U937 cells immediately after receiving hyperthermia at specified times and temperatures.

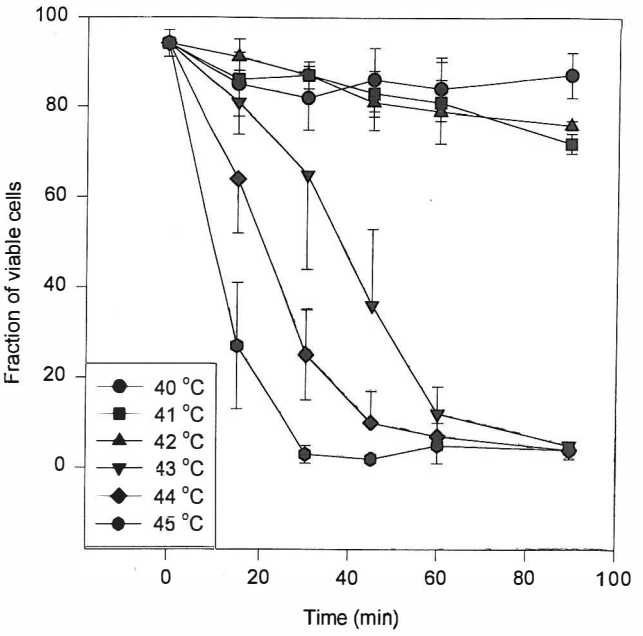

Fig. 2. Fraction of viable U937 cells after 96 hrs of incubation at 37°C after receiving hyperthermia at specified times and doses.

MATERIALS AND METHODS

Cell culture

U937 leukemic cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (prepared freshly in our lab), 120 mg/L penicillin, and 200 mg/L streptomycin. The cells were incubated at 37°C, 7.5% CO_2 and full humidity. The cells were maintained in logarithmic phase of growth by serial subculturing of 2×10^5 - 10^6 cells/mL at 4 day intervals in 25 cm^2 culture flasks (Nunc). Cell viability was determined by trypan blue dye exclusion assay and was expressed as the percentage of cells excluding the dye.[18]

Hyperthermia application

1.2 mL of 2×10^6 U937 cells/mL in RPMI 1640 culture medium without FCS[19] were exposed to immediate HT by immersing them in a thermostated water bath (Haake F3) with ± 0.1°C precision. Heat was applied in the range of 40-45°C for different intervals of 15, 30,45, 60 and 90 minutes. After heat application the samples were allowed to stand for 5 minutes at room temperature before further incubation.

After determination of cell viability 1 mL of the cells was transferred to 25 cm^2 culture flasks (Nunc) containing 9 mL of the culture medium such that each flask ultimately contained 2×10^5 cells/mL in RPMI 1640 supplemented with 10% FCS. The cells were incubated for a further 96 hours and then used for various assays.

NBT reduction assay

The NBT solution was prepared as 1 mg/mL in 0.01 M of phosphate buffered saline, pH = 7.4. Then 0.1 mL of heat treated U937 cells–prepared as described above–containing 5×10^4 cells in RPMI 1640 supplemented with 20% FCS was mixed with 0.1 mL of freshly prepared NBT solution and incubated for 45 min at 37°C. Cells were then maintained at 4°C. A minimum of 200 cells were counted using a

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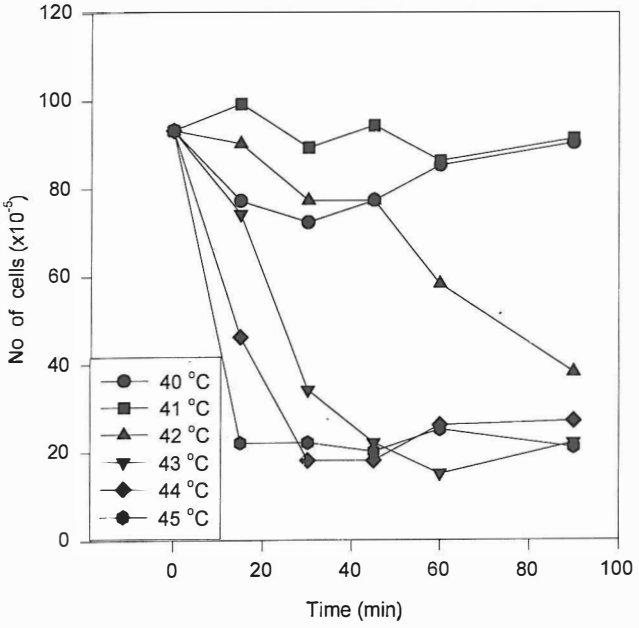

Fig. 3. Number of U937 cells after 96 hrs of incubation at 37°C after receiving hyperthermia at specified times and doses.

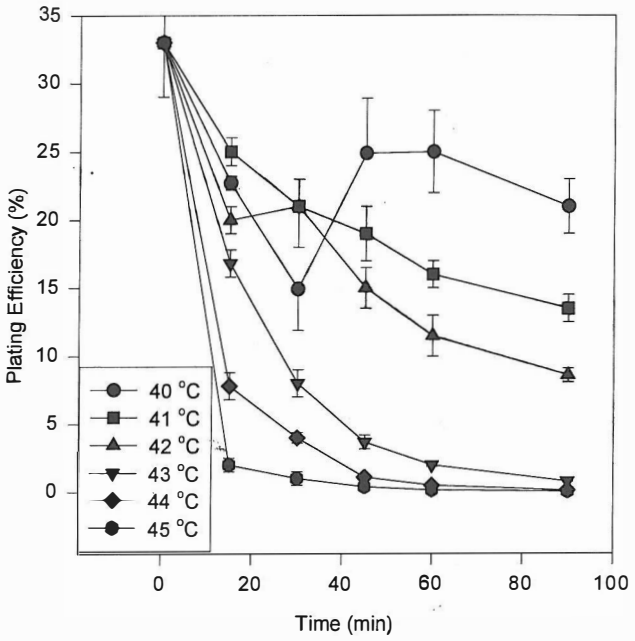

Fig. 4. The plating efficiency (PE) of U937 cells after treatment with hyperthermia at various temperatures and times.

hemocytometer and the fraction of differentiated cells capable of reducing NBT and producing a dark blue precipitate of nitro blue diformasan (NBD) was determined.[20,21]

Latex particles phagocytosis assay

Differentiated U937 cells were assayed for their ability to phagocytize protein coated latex particles according to a modification of Shaala et al.[22] A protein coated latex particle

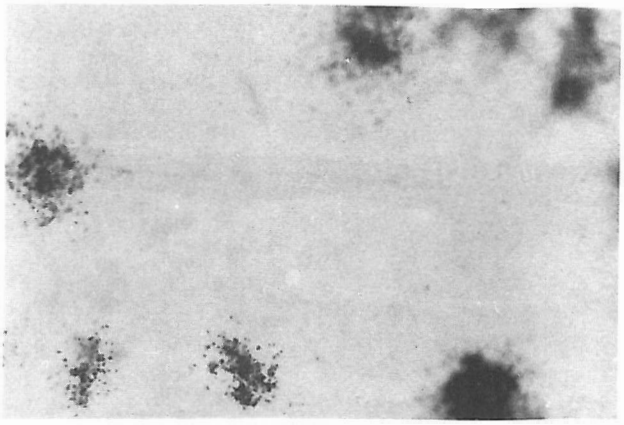

Fig. 5. Colonies produced by U937 cells in semi-solid agar cultures (×100).

suspension marketed commercially as a pregnancy test (Ortho Gravindex) was used for the assay. The commercial suspension of particles was diluted 1:10 with PBS and 0.1 mL of the diluted suspension was mixed with 0.1 mL of RPMI 1640 supplemented with 20% FCS and containing 5×10^4 HT U937 cells and then incubated for a further 60 min. Finally cells were centrifuged at 1000 g for 20 min at 4°C. The cells were washed twice with PBS and resuspended in 0.2 mL of PBS. At least 200 cells were counted for latex particle phagocytosis positive cells (a cell containing a minimum of 10 particles was considered a positive cell).

Fluorescent antibody assessment test

The ability of differentiated U937 cells to bind to an antibody raised against mature alveolar macrophages of mice was used as a criterion of differentiation. The production and conjunction of this antibody to fluorescein isothiocyanate (FITC) has been described before.[23] For this assay 5×10^4 HT treated U937 cells were washed 3 times with PBS and then incubated with 0.1 mL of FITC-Ab for 45 min at 4°C. Then the cells were washed 3 times with PBS and counted for FITC positive cells under a fluorescence microscope.

Cytological studies

Microscopic slides of U937 cells were prepared by cytocentrifuge (Cytospin, Shandow). The slides were stained with Wright-Geimsa (Merck) and observed under a light microscope.

Colony formation assay

1×10^3 U937 cells treated with HT were cultured in RPMI 1640 supplemented with 0.3% agar (Difco) and 20% FCS in 30 mm Petri dishes (Nunc) and incubated for 10 days in the above condition. Colonies were stained with Wright stain and counted. A minimum of 50 cells was considered as a colony. The plating efficiency was calculated as:
PE=(Number of colonies)/ (Number of cells plated)×100

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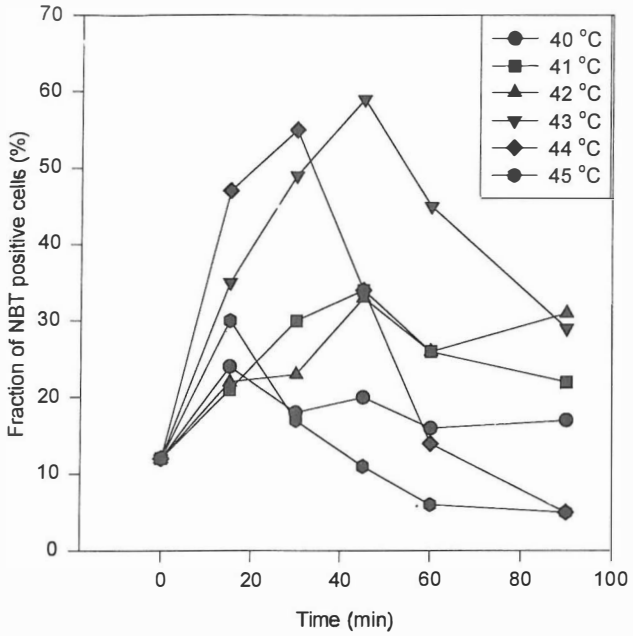

Fig. 6. Fraction of U937 cells which could reduce NBT after 96 hrs of incubation at 37°C following treatment with hyperthermia at various temperatures and exposure times.

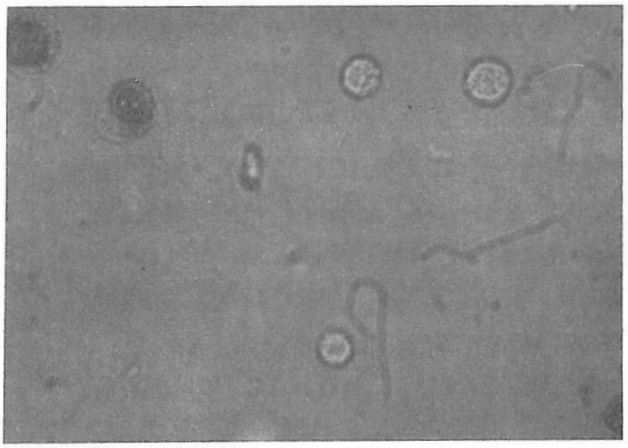

Fig. 7. Photomicrograph of NBT-positive U937 cells (x250).

All experiments described in various sections above were repeated 3 times.

RESULTS

HT caused both short and long term damage in U937 cells. Short term damage was expressed as a decrease in cell viability immediately after HT. As indicated in Figure 1, viability decreased to 70% of the control after 90 min of heating at 45°C. Long term damage was expressed when the cells were incubated at 37°C for 96 hrs after HT. As shown in Figure 2, 45 minutes of heating at 43°C dropped the viability to 30% of the control and 15 min at 45°C was

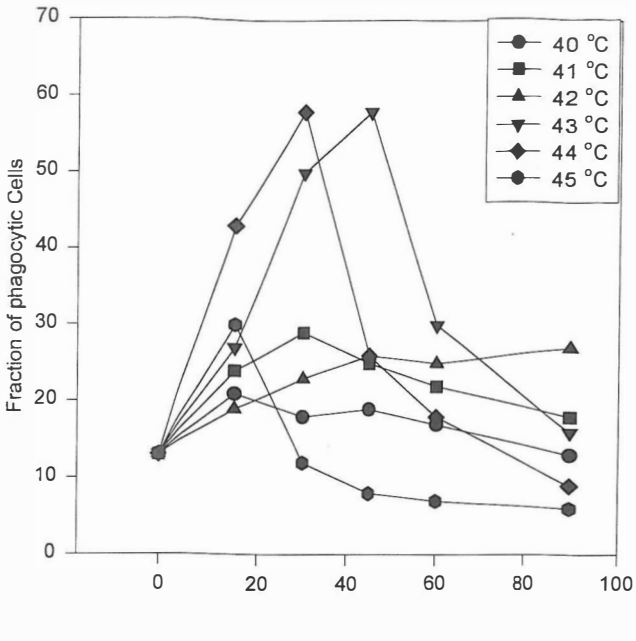

Fig.8. Fraction of U937 cells which could phagocytize latex particles after 96 hrs of incubation at 37°C following treatment with hyperthermia at various temperatures and exposure times.

enough to drop the viability to about 20% of the control. The actual counts of heat treated U937 cells after 96 hrs of incubation are shown in Figure 3.

The alteration in the plating efficiency after heat treatment is shown in Figure 4 as a function of the heating time. At temperatures below 43°C at which the cell count did not drop significantly there was a reduction in the plating efficiency of heat treated U937 cells. At 43°C and higher temperatures there was a sharp decrease in the plating efficiency similar to that observed for the total cell count. Figure 5 shows a photograph of the colonies produced by the HT U937 cells.

The result of HT on U937 cells to produce O^- and reduce NBT is shown in Fig. 6. Two distinct effects of HT on these cells are evident from the heating curves. The initial rise in the curves at low doses or exposure times indicated that HT has caused differentiation of U937 cells towards mature macrophages which were capable of reducing NBT. The decline in the final phase of the heating curves is an indication of the cytotoxic effect of HT. It can be seen that 30-45 min of heat treatment at 43°C has induced the highest rate of differentiation in U937 cells. Figure 7 shows a photograph of NBT positive cells.

Figure 8 shows some of the U937 cells which have been treated with hyperthermia and then allowed to phagocytize latex particles. Only 10% of untreated control U937 were able to phagocytize latex particles. However, heat treatment at 42°C for up to 90 min, 43°C for 15-45 min and 44°C for 15-30 min increased the fraction of cells which could

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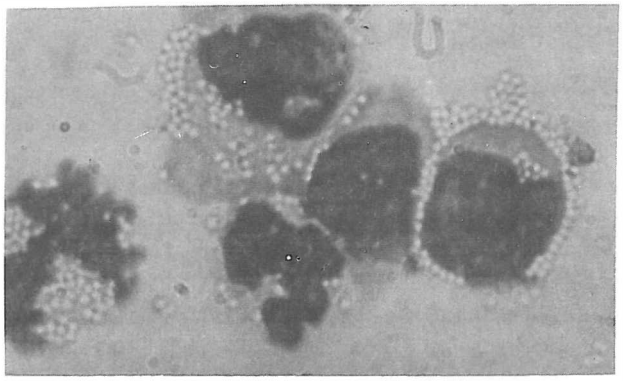

Fig. 9. Photomicrograph of U937 cells which have phagocytized latex particles (×1000).

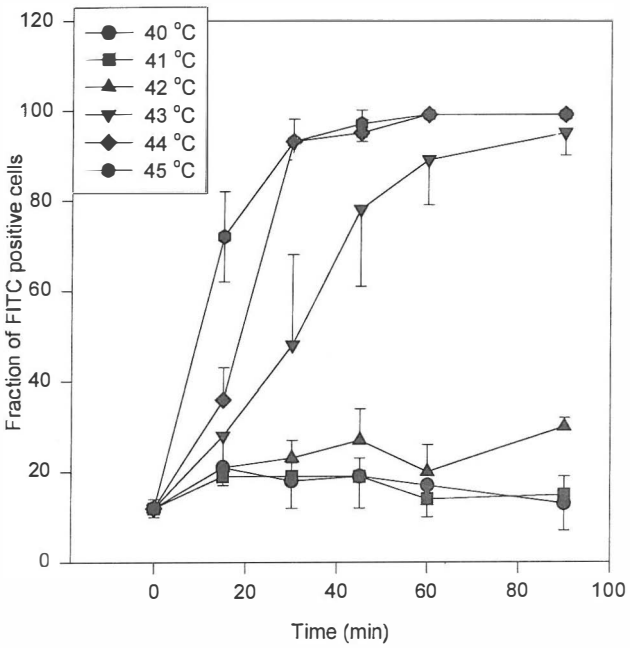

Fig. 10. Fraction of U937 cells which showed positive reaction with FITC conjugated anti-macrophage antibody after 96 hr of incubation at 37°C following treatment with hyperthermia at various temperatures and exposure times.

phagocytize latex particles. Lower temperatures were not effective and 15 min at 45°C was slightly effective. Figure 9 shows a photograph of heat treated U937 cells which have phagocytized a large number of latex particles.

The interaction of FITC conjugated anti-macrophage antibody with U937 cells treated with hyperthermia is shown in Figure 10. Heat treatment at 42°C increased antibody binding by the cells in a dose dependent manner. Lower temperatures were almost ineffective. At higher temperatures there was a sharp increase up to 100% in the fraction of FITC positive cells. A photomicrograph of FITC positive cells is shown in Figure 11.

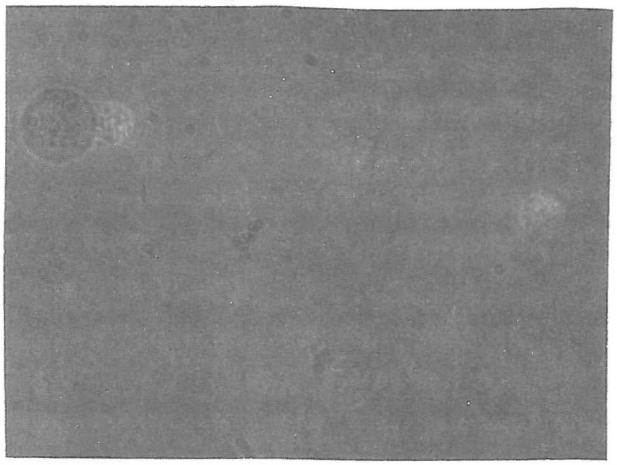

Fig. 11. Photomicrograph of FITC positive U937 cells. Cells were treated with hyperthermia at various temperatures and exposure times, incubated at 37°C for 96 hrs, and treated with antibodies as described in the methods section (×400).

DISCUSSION

In the present work we have investigated the effects of hyperthermia on the proliferation and differentiation of U937 cells. Our results showed that mild hyperthermia could induce differentiation in U937 cells in a dose dependent manner. Various experimental criteria were used to assess the differentiation of U937 cells. For each criterion employed there appeared to exist a specific and distinct dose response curve and an optimum exposure time for the induction of differentiation. Such a phenomenon has been observed and reported for other compounds inducing differentiation in leukemic cells.[24,25]

Inducers of differentiation in leukemic cells have been classified as partial or strong inducers according to the degree of differentiation they induce in their target cells.[26] Accordingly, hyperthermia treatment at temperatures of 42 and 43°C and exposure times of less than 45 minutes were considered as strong inducers. Lower temperatures or shorter exposure times could only partially induce differentiation, whereas at higher temperatures or longer exposure times the viability was reduced drastically and overshadowed the differentiating effect of hyperthermia in U937 cells.

Previous reports with HL60 cells had shown that hyperthermia treatment at temperatures between 42.5 and 43.5°C for 1 hr was most effective in induction of differentiation in those cells.[27] However, in our studies this dose of heat reduced the viability of U937 cells to less than 30%. The difference might be due to the difference in biological heat resistance and therefore the amount of thermal energy required to induce differentiation in the two cell lines. It might be considered that the partial induction of differentiation in these cells by mild hyperthermia is merely

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a consequence of heat induced arrest of proliferation. However, the results of experiments with Acivin, an antagonist of glutamine, has shown that it could inhibit proliferation of U937 cells without triggering their differentiation.[28]

The three criteria used to evaluate the extent of differentiation, namely NBT positive reaction, latex particle phagocytosis and FITC positive reaction, gave fairly similar and consistent results at low temperatures or short exposure times at temperatures above 43°C. However, at exposure times longer than 30 min at high temperatures there was a sharp increase in the fraction of FITC positive cells up to 100% (Fig. 10), while there was a decline of NBT positive and phagocytic cells under similar conditions (Figs. 6 and 8). The difference can be partly described on the basis of the nature of the mechanisms of these reactions. At high temperatures the permeability of cell membranes increases; therefore there is influx of FITC-Ab particles into the cells. These cells are considered as FITC positive cells. This is similar to results previously reported on the effect of hyperthermia on phagocytosis of FITC- dextran particles by tumor cells.[29] On the other hand, production of O_2 and conversion of NBT to blue-black particles of nitro blue diformasan occurs only in viable cells.[21]

Hyperthermia has been widely used as an anti-cancer agent. There is some evidence that leukemic cells are more heat sensitive than their normal bone marrow progenitors or stem cells.[30] The reason for this difference in heat sensitivity has been attributed to several parameters including the state of differentiation, chromosomal abnormalities, presence and function of several oncogenes, and the type of leukemia.[31] This increased heat sensitivity of leukemic cells with respect to normals has been the rational basis of using hyperthermia to purge leukemic cells from bone marrow specimens.[32] It has also been possible to use whole blood hyperthermia during bone marrow transplantation to destroy residual leukemic cells.[33]

The results we have presented here show that hyperthermia can be used as a differentiation inducing agent in leukemic cell lines. The extent of differentiation depends on the temperature and exposure time. This is a reasonable and promising alternative to cytotoxic properties of hyperthermia used in the purging experiments.

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