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Lack of Development of Thermotolerance in Early Progenitors of Murine Bone Marrow Cells[1]

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ABSTRACT

We have studied the sensitivities of four hematopoietic stem cell types to heat stress as well as their abilities to develop thermotolerance. Granulocyte-macrophage colony forming units were the most heat resistant bone marrow progenitors tested. Of the erythroid progenitors tested, erythrocyte colony forming units were more resistant than the two more primitive erythrocyte burst forming units.

To determine their ability to develop thermotolerance, hematopoietic precursors were heated *in vivo* at 43°C for 30 min. At various times thereafter the hematopoietic stem cells were flushed from female C3Hf/Sed mouse preheated tibia. The bone marrow cell suspensions were then heated *in vitro* and plated for colony formation. The four stem cell precursors differed markedly in their abilities to develop thermotolerance. The thermotolerance induced in granulocyte-macrophage colony forming units reached a maximum at 3–6 h after heating and disappeared by 24–48 h. The thermotolerance in erythrocyte colony forming units (0.5 units erythropoietin/ml media) reached a maximum at 3–6 h and disappeared by 48–72 h. The maximum level of thermotolerance reached by granulocyte-macrophage colony forming units and erythrocyte colony forming units was approximately the same. On the contrary, the two more primitive erythrocyte precursors which were grown by the addition of 2.5 and 5 units erythropoietin/ml of media do not develop thermotolerance.

INTRODUCTION

Mammalian cells when exposed to a nonlethal heat shock have the ability to acquire a transient resistance to one or more subsequent exposures at elevated temperature. This phenomenon has been termed thermotolerance (1, 2). Recent studies indicate that thermotolerance can be induced not only in mammalian cell lines but also in certain tumors (3–5) and normal tissues (6–10).

Studies on the thermal response of bone marrow cells after single or multiple heat treatments have intrinsic biological interest. Bone marrow contains pluripotent stem cells; these stem cells have the ability to divide and give rise to differentiated progeny at some stage of their development. Recently evidence has been accumulating that shows that some proteins involved in differentiation may also be HSPs[3] (11–14). Tissue culture techniques have been developed to assay selectively progenitor populations in each of the major lines of blood cells. Using these assays, one can study the effect of hyperthermia on progenitor cells in different blood cell lineages.

In addition, the information to be obtained may be of clinical value. If patients whose bone marrow has been severely compromised by previous treatments such as chemotherapy are treated by hyperthermia, further bone marrow suppression might become a treatment-limiting factor. Furthermore when whole body hyperthermia is combined with chemotherapy, bone marrow toxicity plays a dose-limiting role.

In this report, we have studied the effects of hyperthermia on the induction and development of thermotolerance in mouse bone marrow progenitors, specifically CFU-GM, CFU-E, and BFU-E. Our data show that very primitive precursors do not develop thermotolerance but as differentiation proceeds, the ability to develop thermotolerance is acquired.

MATERIALS AND METHODS

Mice. Eight- to 12-wk-old female C3Hf/Sed mice were housed 4 to a cage and given standard lab chow and acidified water *ad libitum*.

Marrow Cell Preparation. Bone marrow cell suspension were prepared by flushing the tibia with MEM supplemented with 15% FCS. The cell suspension was then counted using a hemocytometer and centrifuged at 1000 rpm for 12 min. The resulting pellet was resuspended and diluted to approximately 1×10^6 cells/ml in MEM plus 15% FCS for further experiments.

***In Vitro* Heating.** The cell suspensions in MEM supplemented with 15% FCS were heated in 15-ml centrifuge tubes (Corning Glass Works, Corning, NY) and immersed in a precision temperature controlled circulating water bath (Precision Scientific Group). The warm-up time for the cell suspension in a 15-ml centrifuge tube was less than 1 min. The pH of the medium was maintained at 7.4 during heating by preflushing the cell suspension with 5% CO_2 and 95% air before sealing the tubes. After heating, the cell suspensions were centrifuged and resuspended in α-MEM before plating (see below).

***In Vivo* Heating.** For *in vivo* heating, the mice were anesthetized with sodium pentobarbital (0.06 mg/g body wt) (Diamond Laboratories, Inc., Des Moines, IA) and one leg of the animal was immersed in a circulating hot water bath at preset temperature. The temperature of the tibia was approximately the same as that of the water bath. However, a few minutes were required for the tibia temperature to reach the water bath temperature.

Cell Survival Determination. To measure the survival of erythrocytes and granulocyte-macrophage progenitors, the methods of Van Zant *et al.* (15) were used with minor modifications. Erythrocyte progenitors

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[3] The abbreviations used are: HSP, heat shock protein; CFU-GM, colony forming unit, Granulocyte and Macrophage; CFU-E, colony forming unit, Erythrocyte; BFU-E, burst forming unit, Erythrocyte; EPO, erythropoietin; MEM, minimal essential medium; FCS, fetal calf serum; HSP 70, heat shock protein with a molecular weight of 70,000.

were grown in α-MEM containing 0.8% methylcellulose (Sigma Chemical Co., St. Louis, MO), 30% heat inactivated fetal calf serum (Hyclone, Logan, UT) and 1% deionized bovine serum albumin (Sigma). In addition, 10^{-4} M β-mercaptoethanol was used to increase burst forming activity, and 0.5 to 5 units of erythropoietin (Connaught Laboratories, Ontario, Canada) per ml of medium was used for colony formation of erythrocyte precursors at different levels of differentiation. The bone marrow cells containing erythrocyte precursors were plated in 35-mm Petri dishes containing 1 ml each of medium and were incubated at 37°C with 5% CO_2 and 98% humidity. CFU-Es were counted in 48 h after plating (16). Each colony containing 8–64 cells was counted as one colony. BFU-E (2.5 or 5 units of erythropoietin) were counted on day 8 and each colony contained at least 50 cells. In all cases erythroid colonies were stained with benzidine dihydrochloride (Sigma) and scored at ×40–×100 under a dissecting microscope and scored in the first 5 min of staining. The colony forming efficiency of CFU-E, the most differentiated erythroid progenitors, was 240 ± 30 (SD)/10^5 nucleated cells, that of BFU-E (2.5 units erythropoietin/ml) was 27 ± 4/10^5 nucleated cells, and that of BFU-E (5 units of erythropoietin) was 9/10^5 nucleated cells. BFU-E, which can be grown with 5 units of erythropoietin/ml medium, is less differentiated than the BFU-E, which can be grown with 2.5 units erythropoietin/ml medium.

CFU-GMs were grown in α-MEM containing 30% heat inactivated fetal calf serum and 1% bovine serum albumin. In addition, 15% conditioned medium from confluent mouse L929 cultures (10 days old) was used as a source of colony stimulating factor (CSF) and 10^{-4} M β-mercaptoethanol was used to increase burst forming activity. One day before each experiment, 2.5 ml of the above media containing 0.5% noble agar (Difco Laboratories, Detroit, MI) were added to individual 60-mm Petri dishes. Immediately after heat exposure cells were diluted in 2 ml of the above media with 0.3% noble agar and poured into the prepared dishes with the 0.5% noble agar underlayer. The cells were then incubated at 37°C with 5% CO_2 and 95–98% humidity. The colonies were counted on day 8 with a dissecting microscope. Both granulocytes and macrophages were counted separately and then summed together for survival calculations of granulocyte-macrophage progenitors (CFU-GM). Only colonies containing 50 or more cells were scored. The colony forming efficiency of CFU-GMs was 200 ± 20/10^5 nucleated cells for unheated controls. Surviving fraction was defined as the clone forming efficiency of heated marrow cells divided by that of unheated controls. The concentration of cells was adjusted from 1×10^5 cells/dish for controls to 2.5×10^6 cells/dish according to the given heat dose. Although colony number varies linearly with the number of marrow cells plated (15, 16), the growth is independent of the cell number in the range used in all experiments. On the average, 3 plates/point were used. All experiments were performed at least twice and yielded consistent results. The variation between each point in different experiments was approximately ±10%. The range of colonies counted for CFU-GMs and CFU-Es was from 5–200 and that of the two BFU-Es was 6–60 for BFU-E (2.5 units of EPO) and 3–50 for BFU-E (5 units of EPO).

RESULTS

Effect of Single Heat Treatment on CFU-GM, CFU-E, and BFU-E. Bone marrow suspensions (1×10^6 cells/ml) were heated *in vitro* for various times at 41–44°C. The heat responses of different bone marrow progenitors are shown in Chart 1. It is clearly demonstrated that CFU-GMs are more heat resistant than are CFU-Es and BFU-Es. For example, 180 min of heating at 42°C reduced the survival of CFU-GM to 3×10^{-1}, that of CFU-E to 8×10^{-2}, and that of BFU-E to 4×10^{-3}. There was evidence of thermotolerance development in CFU-GM and CFU-E with prolonged heat treatments at 41 or 42°C (data for up to 180 min heating only are shown). Similar differences in heat sensitivity were observed at higher temperatures; *e.g.*, at 43°C, 40 min heating reduced survival of CFU-GM to 10^{-1}, that of CFU-E to 5×10^{-2}, and that of BFU-E (2.5 units EPO/ml of media) to 1×10^{-2}. However, the differences in heat resistance is less obvious for temperatures above 42°C because of the steepness of the survival curves.

Effect of Fractionated Heat Treatment on CFU-GM, CFU-E, and BFU-E. In this series of experiments, the tibia of the anesthetized mice were first heated to 43°C for 30 min. This heat treatment *in vivo* reduced the survival level of BFU-Es to approximately 50% but did not reduce CFU-GM or CFU-E survival. Surviving fractions were always corrected for the initial cell killing resulting from the first *in vivo* heat treatment in all split-dose experiments by using the surviving fractions resulting from the first treatment as 1. After the first priming heat treatment *in situ*, some of the mice were sacrificed immediately for the zero-h time point while the others were returned to their cages for various times up to 72 h before being sacrificed. Marrow cell suspensions were prepared from these preheated mice and challenged by graded heat treatments at 43°C *in vitro*.

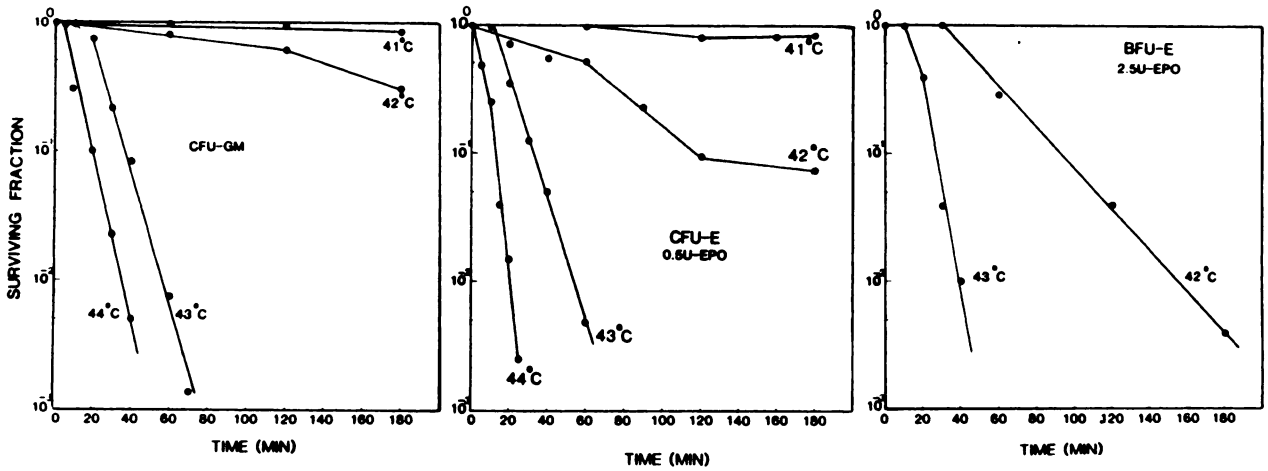

Chart 1. Heat response of CFU-GM, CFU-E, and BFU-E. In this series of experiments, bone marrow from tibia was heated at different temperatures *in vitro*. The survival was then determined on soft agar for CFU-GM and on methyl cellulose for erythrocyte precursors.

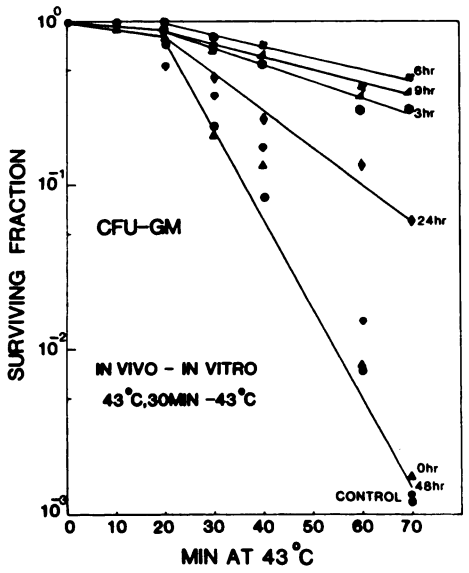

Chart 2. Thermotolerance in CFU-GM. In this experiment, tibia of the anesthetized mice were heated *in vivo* at 43°C water bath for 30 min. The mice were then returned to their cages and at various times thereafter the mice were sacrificed, tibia were flushed, and nucleated bone marrow cells were counted and heated at 43°C for various times. The cells were then plated for colony formation. *Control*, mice which did not receive any pretreatment *in vivo*; *0hr*, mice which did receive a dose of 43°C for 30 min *in vivo* and were immediately sacrificed and then treated *in vitro* at 43°C for various amounts of time.

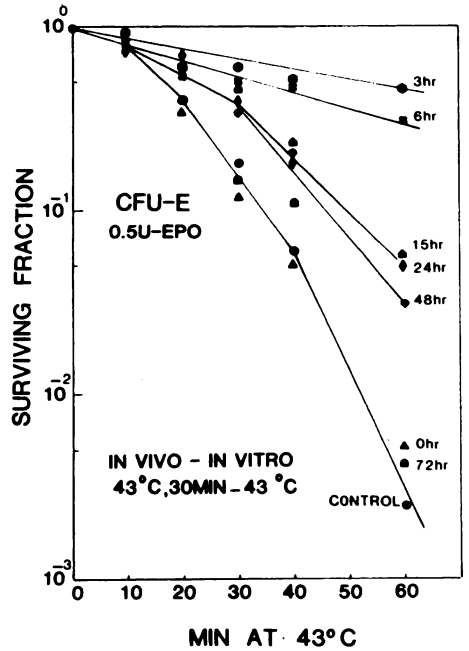

Chart 3. Thermotolerance in CFU-E. The mice were treated as in Chart 2 and bone marrow cells were plated in 0.5 units erythropoietin/ml culture medium for colony formation.

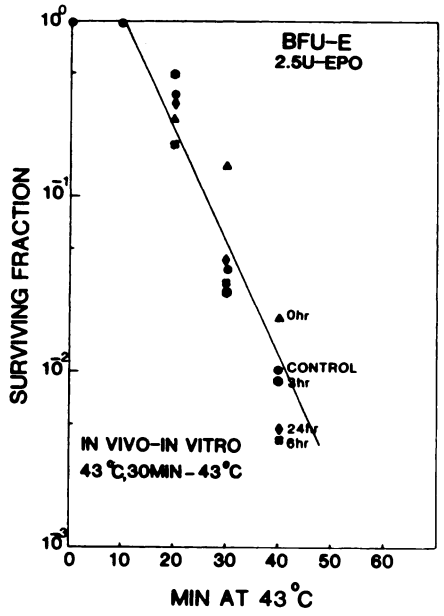

Chart 4. Thermotolerance in BFU-E. The mice were treated as in Chart 2 and bone marrow cells were plated in 2.5 units erythropoietin/ml culture medium for colony formation.

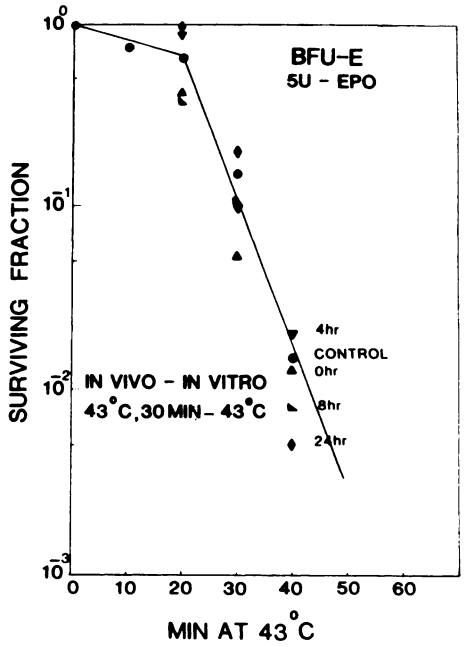

Chart 5. Thermotolerance in BFU-E. The mice were treated as in Chart 2 and bone marrow cells were plated in 5 units erythropoietin/ml culture medium for colony formation.

The results of thermotolerance induction is shown in Charts 2–5. As depicted in Chart 2, CFU-GMs show a dramatic increase in cell survival about 3–9 h after the initial heat treatment. There is a decrease in survival value 24 h afterward and thermotolerance completely disappears by 48 h. CFU-E, the most differentiated erythroid precursor, also shows a dramatic increase in survival 3 h after the initial heat treatment. Thermotolerance as in CFU-GM peaks at around 3 h, decreases gradually, and disappears by 72 h. (Chart 3). Charts 4 and 5 show the effect of fractionated heat treatment on BFU-Es, the two more primitive erythroid precursors. None of the BFU-Es was capable of development of thermotolerance.

Chart 6 shows the kinetics of the development and decay of thermotolerance in different classes of bone marrow progenitors. Our data clearly show that the maximum thermotolerance is reached shortly after the priming heat treatment for both CFU-GM and CFU-Es; however, the tolerance acquired by CFU-GM

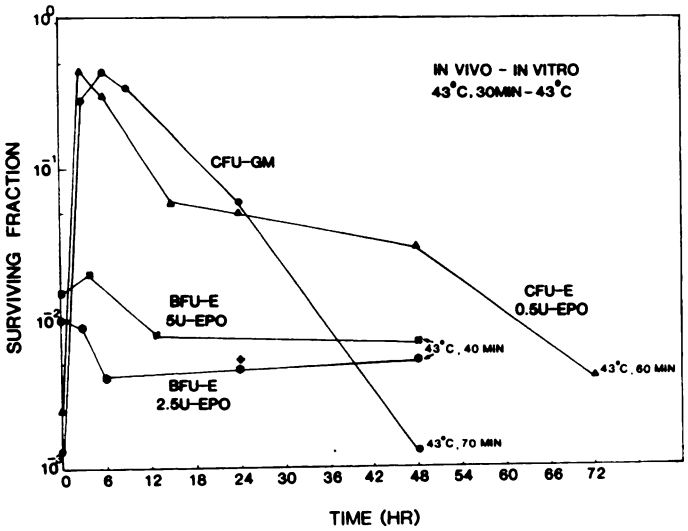

Chart 6. Kinetics of thermotolerance induction and decay in CFU-GM, CFU-E, and BFU-E. The tibia of anesthetized mice were treated *in vivo* at 43°C for 30 min. The mice were then returned to their cages and at various periods of time they were sacrificed and bone marrow was treated *in vitro* at 43°C for 70 min for CFU-GM, 43°C for 60 min for CFU-E, and 43°C for 40 min for BFU-E. The cells were then plated for colony formation. The different heat dose was used for the second treatment to give the same approximate survival value for all progenitors.

decays faster than that of CFU-E. On the other hand, thermotolerance does not develop in BFU-E after the priming heat treatment. The second testing dose of 43°C, 70 min, 43°C, 60 min, and 43°C, 40 min were chosen for CFU-GM, CFU-E, and BFU-E, respectively. The above treatment was designed so as to yield approximate isosurvivals for control bone marrow not receiving the first *in vivo* pretreatment.

DISCUSSION

The induction of thermotolerance with either chronic heating at nonlethal temperatures for prolonged periods of time or by split dose heat treatments has been shown in various mammalian systems (1–10). Here we have shown that thermotolerance develops in two classes of bone marrow progenitors, namely CFU-GM and CFU-E. These two progenitors not only show thermotolerance with prolonged heating at 41 and 42°C but they can also develop thermotolerance with split-dose heat treatments. The maximum thermotolerance observed is as early as 3 h after heating. Thermotolerance decays more rapidly than that found in most other mammalian systems. In CFU-GM thermotolerance disappears between 24 and 48 h. The kinetics of thermotolerance induction of CFU-Es, which are the highly differentiated erythroid precursors, is similar to that of CFU-GM. Thermotolerance in CFU-E disappears completely 48–72 h after the priming heat treatment. Differences in decay rates may be due to the fact that the damage sustained by CFU-GM is less than that of CFU-E with the priming heat dose (*i.e.*, 43°C for 30 min) used in these series of experiments. In contrast, the two more primitive erythroid precursors are more sensitive to heat and do not develop thermotolerance.

There are no data available on the thermotolerance induction in hemopoietic progenitors. However, there are two reports in the literature, one by Van Zant *et al.* (15) in which he studied the heat response of various bone marrow progenitors. The results reported are up to 180 min of continuous heating at 41 and 42°C. Since thermotolerance usually develops after 4 h, it is not possible to deduce any conclusions from that study. The other available data are by Bromer *et al.* (17) in which continuous heating at 41°C was used on human cell colony forming units over a 24-h period. The results of that report do not show any thermotolerance with chronic heating. Split-dose heating was not studied in that report.

According to our previous report (18), we did observe thermotolerance with chronic and split-dose heat treatments in CFU-GM. These split-dose heat treatments were done by allowing the thermotolerance to develop both *in vivo* and *in vitro* and the results were similar. The above observation indicated that for at least the first 24 h there was no problem as far as bone marrow regeneration. Other reports (19) have shown that there is no change in the percentage of early myeloid, metamyelocytes, segmented neutrophils, or erythroblasts for 7 days around the temperature range used in our studies. They also showed no significant change for the number of CFU-GM over a 7-day period.

The induction of thermotolerance has been shown to correlate with the synthesis of heat shock proteins, specifically HSP 70 (20). The absence of this protein has been demonstrated in various early stages of embryonic systems. For example, in sea urchin embryos heat shock protein is not synthesized at any stage prior to the blastula stage (11). Similarly in *Drosophila*, no HSP is synthesized up to blastoderm stage (12). Undifferentiated teratocarcinoma stem cells also do not express heat shock genes (14). In mouse embryo, HSP 70 is not synthesized at a detectable rate and thermotolerance is not expressed at the one-cell stage after heat shock, whereas blastocysts have the ability to synthesize HSP 70 and to develop thermotolerance (13, 21). One-cell embryos are more sensitive to heat than are blastocysts (21). A recent report on human erythroid cells (22) indicated that preincubation of these cells with hemin induces erythroid maturation and causes the accumulation of a M_r 70,000 protein which has been found to be the same as HSP 70. This protein was absent in untreated cells.

In this study we have demonstrated that in bone marrow cell populations, as differentiation proceeds, not only do cells become more heat resistant but they also acquire the ability to develop thermotolerance. Although there is an elevated level of HSP 70 in the general bone marrow populations (18), we cannot be certain that the heat-induced HSP synthesis in total marrow population accurately represents the profiles of protein synthesis of any class of marrow progenitors.

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