Potentiation of Differential Hyperthermic Sensitivity of AKR Leukemia and Normal Bone Marrow Cells by Lidocaine or Thiopental

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Previous work has utilized spleen colony formation to evaluate the fractional survival of AKR leukemia and normal bone marrow cells after *in vitro* heat exposure. An inherently greater sensitivity of neoplastic cells to thermal killing, as compared to normal syngeneic stem cells, has been established both at 41.8°C and 42.5°C. Normal bone marrow colony-forming units were assayed in lethally irradiated (750 cGy) mice. Leukemic colony-forming units were assayed in nonirradiated mice. Using this methodology, the authors demonstrated that the differential effect of hyperthermia on AKR murine leukemia and AKR bone marrow cells can be further enhanced by the addition of lidocaine or thiopental to incubation mixtures. These findings may have application to autologous bone marrow transplantation in humans.

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FOR PATIENTS REQUIRING a bone marrow transplant, infusion of autologous bone marrow obviates graft-*versus*-host as well as graft rejection problems due to histoincompatibility of allogeneic marrow. When the bone marrow transplant is used as treatment for a patient with acute leukemia, however, the remission marrow may contain undetected leukemic cells.[1] A technique for purging the remission marrow of any leukemic cells would allow autologous marrow to be used to rescue leukemic patients given ablative chemo-radiotherapy without reinfusing the leukemia cells this therapy is trying to eliminate. It has been speculated that human leukemia cells may be more sensitive to hyperthermic killing than normal hemopoietic cells.[2,3,4] Both with L1210[5] and AKR murine leukemias[3] *in vitro* heating temperature $\geq$ 41.8°C appears to selectively destroy leukemia cells.

In several model systems, hyperthermic killing of neoplastic cells can be potentiated both *in vitro*[6–8] and *in vivo*[9–12] by anesthetic agents. The data presented herein demonstrate that the presence of lidocaine or thiopental during *in vitro* hyperthermia increases the thermal killing of AKR leukemia cells to a greater extent than it increases the killing of AKR normal bone marrow cells. As a result, a clinically relevant therapeutic index is obtained.

Materials and Methods

Mice

Female AKR mice, 6 to 7 weeks old, weighing 20 to 25 g, were purchased from Cumberland Farms (Clinton, TN). Animals were housed in a climate- and light-controlled environment with free access to food and water.

Transplanted Cell Line

The transplanted cell lines used arose from a spontaneous leukemia.[13] This AKR model is nonimmunogenic in syngeneic mice.[14] The ascites line (provided by Dr. Rex Risser, McArdle Laboratory, University of Wisconsin-Madison) was adapted to a spleen line by intravenous

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injection by tail vein of a spleen perfusate with a new passage every 7 to 10 days (see below). After ten passes, the adaptation as a splenic line met our needs.

Leukemia Cell Preparation

The medium used consisted of one part newborn bovine serum and nine parts of a medium consisting of minimal essential medium (Eagle's) modified with Earle's Salts and with HEPES buffer but lacking bicarbonate and glutamine (Flow Laboratories, McLean, VA). Glutamine (Flow Laboratories) was added on the day of the experiment (292.3 mg/l). The HEPES buffer, in addition to the phosphate buffer already present, provided a stable pH at all temperatures and time points under the conditions of our studies.

The spleen from a leukemic AKR mouse was removed and weighed. Spleens weighing more than 0.3 g were used for preparation of a leukemic cell suspension. Ten milliliters of cold medium (2°C to 4°C) were forced into the spleens by a 10-cc syringe with a 26-gauge needle. Spleens were then teased apart with the aid of forceps. The cell suspension obtained was then transferred to a 50-ml centrifuge tube (2070 Falcon, Oxnard, CA). Cell clumps and spleen fragments were allowed to settle, the upper portion (9 ml) removed by pipette and the suspension was centrifuged at 2°C, 1200 rpm for 10 minutes (RC-3 Sorval, New Town, CT). The supernatant was discarded and the pellet resuspended in 10 ml of medium. An aliquot of the single cell suspension was diluted 1:20 in Turks solution (2% glacial acetic acid in distilled water), and the concentration of nucleated cells was determined from a hemacytometer count. The suspension was then diluted to 10^6 cells/ml using cold medium. The suspension consists almost entirely of leukemia cells.[13]

Bone Marrow Preparation

Tibias and femors were removed from nonleukemic AKR mice. The ends of the bones were clipped and the marrow plugs flushed out with medium using a 26-gauge needle. Plugs were disrupted by repeated gentle aspirations through a 22-gauge needle. The resulting suspension was processed as described above for the leukemic cells.

Spleen Colony Assay

The sensitivity of the assay has been previously demonstrated for both types of cells.[15-18] Cell survival was estimated by the capacity of cells to form macroscopic colonies on the spleen surface of recipient mice 8 or 9 days after tail-vein injection (0.5 ml).

Bone marrow cells for each time point were injected into each of 5 to 10 mice that had been lethally irradiated with 750 cGy, cesium 137 (Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada). Irradiated assay mice were maintained with drinking water containing tetracycline until they were killed. On day 9 the animals were killed; spleens were removed and placed in Bouin's fixative. Grossly visible colonies on each spleen were counted to determine the number of normal colony-forming units (n-CFU) in the initial suspension.[16] A lethally irradiated control group in which no exogenous bone marrow cells were injected was used to estimate the frequency of endogenous colony formation. Bone marrow cells in these experiments had a colony-forming efficiency[13] of 1.5 colony-forming units/10^4 cells.

AKR leukemia cells were similarly assayed, but in nonirradiated mice, which were sacrificed 8 days after injection. These leukemic colony-forming units (L-CFU) had a colony-forming efficiency[13] in these experiments of 100 to 150 colony-forming units/10^4 cells.

We found that the colony-forming efficiency of the leukemic cell line begins to increase after 30 passages. Hence, a supply of cells at passage 19 is maintained in liquid nitrogen (in 10% dimethyl sulfoxide, 30% newborn bovine serum, 60% minimum essential medium Eagle's solution). Aliquots from this stock were used for up to ten passages, *i.e.*, passage numbers 20 to 30.

In Vitro *Incubations*

Ten milliliters of cells (10^6 cells/ml) were incubated inside 50-ml corex tubes (No. 8080-A, Corex, Corning, NY). The tubes were loosely capped with aluminum foil. One tube was used for each time point. The tubes were maintained in ice before they were placed in water baths. These corex tubes allow for temperature equilibration within 4 minutes after placement in water baths.

Time 0 is defined as the time the tubes were placed in Precision Shaking Water Baths (American Scientific Products, McGraw Park, IL) (equilibrated to appropriate temperature ± 0.1°C and covered with 20-mm polypropylene spheres to help maintain constant temperature by reducing evaporation at the water surface) and shaken at 160 oscillations/minute. In addition to mechanical shaking, the tubes were gently swirled by hand every 30 minutes to reduce gradients.

Temperature measurements were made with a 0°C to 50°C (0.1°C) thermometer (No.15-043A; manufactured to meet National Bureau of Standards Monograph #90, Fischer Scientific, Pittsburgh, PA).

At each time point, tubes were removed and placed in ice. To disperse any remaining cell clumps, the cell suspensions were aspirated up and down a 10-ml pipette

ten times. The nucleated cells were then counted and an appropriate dilution made for injection into assay mice as described above.

Analysis of Survival Data

The surviving fraction was estimated as the ratio of the mean spleen colony count at each time point to the spleen colony count at time 0 of a cell suspension without drug. The therapeutic index was calculated as the ratio of the surviving fraction of bone marrow cells at a given time divided by the surviving fraction of AKR leukemia cells exposed under the same conditions.

Drugs

Five minutes prior to heating, drugs were added to cell suspensions, which were maintained in ice.

Four percent lidocaine-HCl (LID) (Astra Pharmaceutical Products, Inc., Worcester, MA) was added such that the final concentration of LID was 0.75 mmol/l. Lyophalized thiopental (Abbott Laboratories, North Chicago, IL) was reconstituted with sterile water before each experiment and an aliquot was taken for each such that the final medium concentrations of thiopental were 0.1, 0.5, or 0.75 mmol/l.

The desired pH of all incubation mixtures was 7.46 ± 0.05. The pH of all incubation mixtures was checked before and after the completion of each experiment. When thiopental was included in the medium, the pH was brought back to the desired pH by the addition of hydrochloric acid (HCl).

Results

The colony-forming ability of leukemic and normal bone marrow cells as a function of time of *in vitro* incubation is illustrated in Figure 1 for 41.8°C and in Figure 2 for 42.5°C. At both temperatures, there was an exponential decrease in L-CFU and n-CFU with time. In both Figures 1 and 2 the terminal slopes of the curves indicate greater hyperthermic sensitivity of the leukemic cells when compared to normal bone marrow cells at both temperatures studied. The addition of LID enhanced the effect of hyperthermia at both 41.8°C and 42.5°C, as is illustrated in Figures 1 and 2, respectively. The effect of the addition of LID on surviving fraction of L-CFU and n-CFU is quantitatively summarized in Table 1, in which the therapeutic index is calculated using data presented in Figures 1 and 2. Therapeutic index increases as the time of incubation increases at 41.8°C or 42.5°C without LID. The difference between therapeutic index found at 120 minutes is only marginally greater at 42.5°C (23.8) then at 41.8°C (15.6).

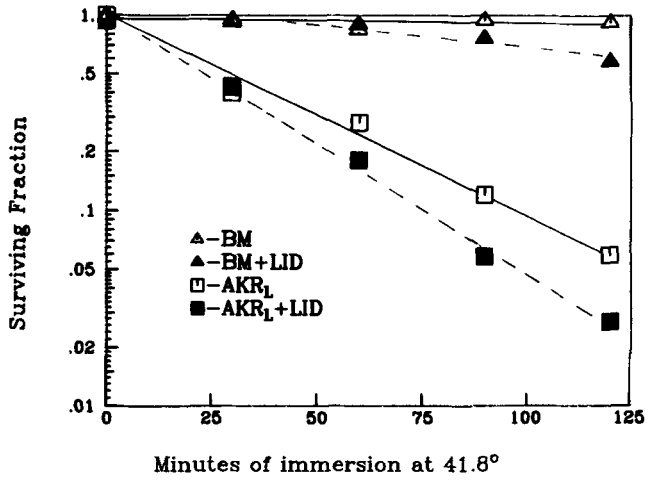

FIG. 1. Effect of lidocaine on survival during 41.8°C hyperthermia. Surviving fraction is estimated as the ratio of spleen colony counts at each time point to the count at 0 time with no drug present. This chart presents surviving fraction for normal bone marrow (BM) (△) and for AKR leukemia cells (AKR$_L$) (□) during heating *in vitro* after immersion in a 41.8°C bath. Closed symbols represent the addition of lidocaine (LID) to cell suspensions. The size of the symbols used is greater than ± one relative standard error of the colony count.

The addition of LID, however, has a substantial effect on the ratio of surviving bone marrow cells and leukemia cells at 42.5°C. In contrast, LID present during hyperthermia at 41.8°C resulted in only modest increases in therapeutic index. Table 2 summarizes data from an experiment in which the surviving fraction of L-CFU and n-CFU 60 minutes after immersion into a 41.8°C

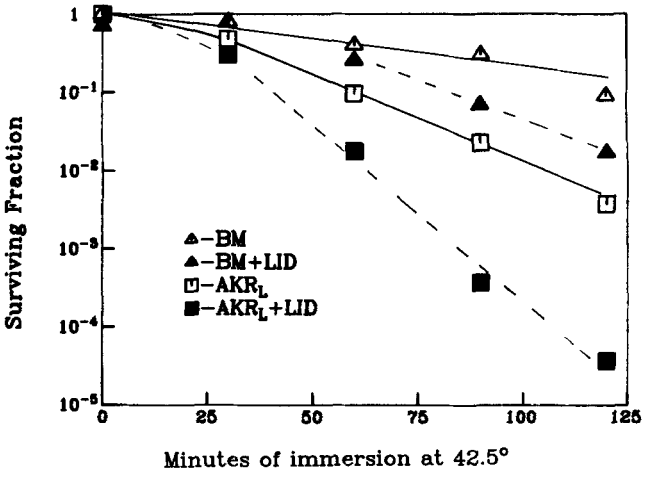

FIG. 2. Effect of lidocaine on survival during 42.5°C hyperthermia. Surviving fraction is estimated as the ratio of spleen colony counts at each time point to the count at 0 time, the time of immersion into the bath, with no drug present. This chart presents surviving fraction for normal bone marrow (BM) (△) and for AKR leukemia (AKR$_L$) (□) during heating *in vitro* at 42.5°C. Closed symbols represent the addition of lidocaine (LID) to cell suspensions. The size of the symbols used is greater than ± one relative standard error of the colony count.

TABLE 1. The Effect of Lidocaine on Therapeutic Index* During Hyperthermia

Time† (min)	41.8°†	41.8° + LID‡	42.5°†	42.5° + LID‡
0	≅1.0	1.0	≅1.0	0.6
30	2.3	2.2	1.6	2.7
60	3.0	4.9	4.1	14.2
90	7.8	13.1	13.2	186.5
120	15.6	21.5	23.8	459.2

* Therapeutic index calculated as ratio of surviving fraction of BM cells at a given time divided by surviving fraction of AKR leukemia cells exposed under the same conditions.

† Time after immersion in water bath.

‡ Temperature of incubation.

§ Lidocaine at a concentration of 0.75 mmol/l added 5 minutes before heating.

LID: lidocaine; BM: bone marrow.

bath are compared at various concentrations of thiopental (0, 0.1, 0.5, 0.75 mmol/l). As the concentration of thiopental increases, there is a rise in TI.

Discussion

The data presented expand our previous work[3] in the AKR leukemia model in which a differential heat sensitivity of normal *versus* neoplastic cells was demonstrated. Our current observation suggests that the addition of certain anesthesic agents to *in vitro* hyperthermic incubations (LID, 41.8°C and 42.5°C; thiopental, 41.8°C) increases the thermal sensitivity of leukemic cells to a greater extent than the increase seen for normal bone marrow cells. Furthermore, this differential sensitivity to LID or thiopental, along with hyperthermia, increases with time of incubation. This effect can be described as synergistic or supra-additive, since neither drug significantly affects the colony-forming ability of normal or leukemic cells at 0°C *in vitro* (See 0 time point, Figs. 1 and 2.) or at 37°C *in vivo*.[19] This same synergistic effect can be demonstrated *in vivo* when treated AKR mice undergo 41.8°C whole body hyperthermia[19] using a radiant heat apparatus.[20]

TABLE 2. Effect of Thiopental Concentration on Surviving Fraction and Therapeutic Index After Immersion for 60 Minutes at 41.8°C *in Vitro*

Concentration (mM)	Surviving fraction		Therapeutic index
	L-CU	n-CFU	(n-CFU/L-CFU)
0	0.36 (±0.16)*	0.85 (±0.27)	2.4
0.1	0.26 (±0.16)	0.84 (±0.32)	3.2
0.5	0.104 (±0.08)	0.54 (±0.26)	5.2
0.75	0.008 (±0.019)	0.18 (±0.20)	22.5

* Mean (± standard error of ratio based on counting statistics).

L-CFU: AKR leukemia colony forming units; n-CFU: normal bone marrow colony forming units.

We correlate decreased colony-forming ability with cell death because of our *in vivo* studies in which decreased L-CFU correlated with increased survival, as well as our *in vitro* studies in which decreased colony-forming units correlated with morphologic changes associated with cell death.[21]

Symonds and colleagues have also shown that a differential sensitivity to hyperthermia killing exists between L-1210 leukemia cells and normal marrow cells *in vitro,* using methods comparable to those used in the current study.[5] Their work did not include a study of the effect of anesthetic agents. When they simultaneously heated normal marrow cell with L-1210 cells, the presence of marrow cells increased the thermal sensitivity of L-1210 cells. They postulated that this was due to the release of diffusable factors. We have studied the AKR system in this regard and have found no such phenomena. Kase and Hahn review, in part, other examples of the differential hyperthermic sensitivity of normal *versus* neoplastic cells of the same origin.[22]

We have studied the effect of the addition of both LID (0.75 mmol/l) and thiopental (1.0 mmol/l) during incubation with an AKR leukemia cell suspension (10^6 cells/ml) at 42°C for 60 minutes in one experiment. The combination of thiopental plus LID at time 0 produces an additive effect on the reduction of surviving fraction (data not shown).

The differential sensitivity described here may reside in membrane differences between normal and neoplastic cells.[23-25] This speculation is supported by studies of hyperthermia sensitivities in which correlations have been made with membrane differences and membrane-active agents.[6-12,26]

The data presented in Tables 1 and 2, which demonstrate the increase in therapeutic index when anesthetic agents are added to *in vitro* hyperthermia, support the argument that such an approach may serve as a useful adjunct to clinical autologous bone marrow transplantation in the treatment of acute leukemia. Further exploration of this thesis using human hemopoietic tissue *in vitro* is therefore encouraged.

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