Differential Effect of Hyperthermia on Murine Bone Marrow Normal Colony-forming Units and AKR and L1210 Leukemia Stem Cells

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ABSTRACT

Thermal dose-survival curves for normal hematopoietic and leukemia cells were assessed by spleen colony assays after in vitro heat exposure ranging from 41° to 45°. No effect of 43° heat treatment on the fraction of cells lodging in the spleen was observed. Marked differences in heat sensitivity were observed between normal, L1210, and AKR leukemia cells, the first being less sensitive than were the malignant cells. Furthermore, a greater relative difference between normal stem cells and leukemia cells was observed at lower temperatures. Normal bone marrow cells forced into regenerative activity prior to heat treatment were more heat sensitive than was their undisturbed counterpart, suggesting that noncycling hematopoietic cells are less heat sensitive than are proliferating cells.

INTRODUCTION

Hyperthermia is a promising modality in treating localized or systemic cancers, either alone or in combination with radiotherapy or chemotherapy. However, evidence concerning whether hyperthermia has a selective effect on the killing of malignant cells has been conflicting (5, 11, 14). For solid tumors, it has been demonstrated that the tumor environment (i.e., decreased pH and nutrients) may provide some selective killing of tumor cells over normal cells (9, 12, 20). However, this phenomenon would not be relevant to a disseminated disease such as leukemia. As hyperthermia, particularly whole-body hyperthermia, may become an adjunct to chemotherapy or extended-field radiotherapy in such disseminated diseases, we were interested in comparing the heat sensitivity of clonogenic bone marrow cells and L1210 and AKR leukemia cells of mice. Our results suggest that leukemia cells may, in fact, be more sensitive to hyperthermia than are their normal counterparts and that there may be a therapeutic advantage for hyperthermia against leukemia cells in some therapeutic regimens.

MATERIALS AND METHODS

Mice. Six- to 9-week-old mice were obtained from a specific-pathogen-free breeding facility at Washington University (St. Louis, MO) with foundation stocks provided by The Jackson Laboratory, Bar Harbor, ME. AKR, DBA/2, and C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice were used. Females were used for cell donors and males for colony assays. Mice were housed 5/cage and were provided free access to food and water.

Transplanted Leukemias. AKR leukemia is a transplantable, widely disseminating, lymphocytic leukemia derived from the thymus of a female AKR mouse (3). It is passed weekly in our laboratory from the spleens of tumor-bearing mice which had received approximately 10⁶ leukemia cells 7 days previously. L1210 leukemia is also a widely disseminating leukemia obtained by us in 1971 from the National Cancer Institute. It has been subsequently passaged weekly in DBA/2 mice in a manner identical to that used for the AKR leukemia.

Preparation of Cell Suspensions. Cell suspensions of leukemia cells were prepared from the spleens of leukemia-bearing mice as described previously (32). Briefly, spleens were minced with scissors and passed through 120 mesh stainless steel with α-MOPS medium. Cells were then counted in a hemocytometer, and a concentration of 10⁶ cells/ml was used for the hyperthermia studies.

For bone marrow cells, femoral marrow was removed by flushing femurs with 2 ml of α-MOPS. Concentrations of either 10⁶ or 10⁷ cells/ml were used for normal bone marrow studies. No difference in survival due to cell concentration was observed at those treatments where both concentrations could be assayed.

Colony Assays. LCFU were assayed by the spleen colony method (4). A fraction of the cell suspension to be assayed was injected i.v. in 0.5-ml doses into the tail veins of 8 recipient mice. Eight days later, the mice were sacrificed, their spleens were removed and placed in Bouin’s fixative, and the macroscopic colonies were counted. The concentration of LCFU per ml in the original suspension was determined and expressed as the fractional survival of LCFU relative to the untreated control. The average number of LCFU in the control group was 3.8 to 6.6 x 10⁴ per 10⁶ cells injected for AKR leukemia and 1.1 to 2.7 x 10⁴ per 10⁶ cells injected for L1210 leukemia.

For NCFU experiments, normal hematopoietic stem cells were assayed by the procedure of Till and McCulloch (29). Briefly, a fraction of the cell suspension to be assayed was injected i.v. in 0.5-ml volumes into the tail veins of previously irradiated mice (10 Gy, 133Cs γ-rays). Spleens were removed 9 to 12 days after cell injection, placed in Bouin’s fixative, and the macroscopic colonies enumerated. The number of NCFU per ml in the original cell suspension was determined and expressed as the fractional survival of NCFU relative to the untreated control. The average concentration of NCFU in the control group was 70 to 90 per 10⁶ cells injected.

The fraction of injected leukemia cells lodging in the spleen (f value) was determined by the technique of Bruce and Meeker (3). Briefly, either 10⁶ control or heated leukemia cells were injected into 2 to 3 recipient mice. Two hr later, once cells had lodged in various organs, the spleens of these recipient mice were removed, and appropriate dilutions of cells were again injected into 6 new recipient mice. Spleens were then counted for leukemic colonies 9 to 12 days later. In addition, appropriately diluted cells from the original populations were directly injected into recipient mice for spleen colony assay. From the resultant colony ratios, the f value can be calculated.

Proliferating NCFU. Two techniques were used to obtain proliferating NCFU. Both made use of the fact that in regenerating marrow, following either irradiation or cyclophosphamide treatment, a large fraction of the NCFU are proliferating. In one procedure (30), mice receive a supralethal dose of 10 Gy whole-body irradiation (137Cs γ-rays) and shortly thereafter are given i.v. injections of 5 x 10⁶ normal bone marrow cells from a syngeneic donor. Their femoral marrow is then used 6 days later. In the

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second procedure, mice receive 2 mg of cyclophosphamide i.p., and the bone marrow is harvested 36 hr later.

**Heating of Cells.** Cells were heated by immersion in precision thermostatically controlled water baths (Lacini Bros., St. Louis, MO) and thermally regulated (±0.03°) by proportional controllers (Yellow Springs Instrument Co., Yellow Springs, OH). Temperatures were monitored by mercury-in-glass thermometers calibrated against a reference standard from the National Bureau of Standards. All temperature transitions were performed by rapidly transferring flasks between the precision water baths and a 37°-water bath to insure reproducible temperature transitions (t1/2 about 25 sec). Flasks were sealed with paraffin wax to prevent leakage. The pH was monitored in representative flasks at the cell concentrations used and was found to remain stable between 7.01 and 7.06 at all times. After the heat exposure, appropriate dilutions for the clonogenic assays were made in α-MOPS and injected into recipient mice in 0.5 ml. Survival curves were fit to the equation

\[
\text{Surviving fraction} = 1 - (1 - e^{-D \cdot t^n}),
\]

by least-squares regression to determine \( D_0 \) and \( n \) values.

**RESULTS**

Survival curves of NCFU of B6D2F1 mice heated in α-MOPS at 42°, 43°, and 45° for various times are shown in Chart 1. The survival of cells maintained at 37° for 120 min in α-MOPS is also shown. The average \( D_0 \) values determined for the steepest portions of the curves were 54, 18, and 2.3 min for 42°, 43°, and 45°, respectively. There is some indication of a change in slope after about 4 hr at 42° which could be an indication of developing thermotolerance, as has been shown previously for other cells (25); however, this phenomenon is not statistically verifiable in these experiments.

The survival of L1210 and AKR leukemia cells after heat exposure at various temperatures is shown in Charts 2 and 3, respectively. Thermal sensitivity is fairly similar at 43° and 45°; however, a difference appears mainly in the shoulder segment of the survival curves with L1210 cells having a much wider shoulder than do AKR cells. Table 1 gives a comparison of \( D_0 \) and \( n \) values of the cell lines investigated. In general, the leukemia cells appear more sensitive to hyperthermia under the experimental conditions used. Furthermore, a greater difference in heat sensitivity between leukemia and normal bone marrow cells is observed at lower temperatures.

Because of the known membrane-modifying ability of heat (13), a possibility existed that hyperthermia may decrease the ability of leukemia cells to lodge in the spleen, which would be mistakenly interpreted as cell killing by our assay. We examined this possibility by determining the fraction of heat-treated tumor cells lodging in the spleen (\( f \) value) of heated AKR leukemia cells.

The \( f \) value was determined as described previously (3). Unheated AKR leukemia cells yielded an \( f \) value of 0.020 ± 0.011 (S.E.). Leukemia cells exposed to 43° for 20 min (surviving fraction = 2 × 10^-3) yielded an \( f \) value of 0.021 ± 0.006. These results demonstrate that heat treatment does not appear to alter the proportion of leukemia cells which lodge in the spleen, thereby showing the validity of the spleen colony assay to measure the survival of heat-treated cells.

Different mouse strains were used for the leukemias and normal bone marrow studies shown in Charts 1 to 3. In order to determine if mouse strain differences could account for the effects observed, a comparison was made of survival after heating at 42° for 3 mouse strains. As shown in Chart 4, no significant differences in thermal sensitivity were observed. These results indicate that the differences in heat sensitivity between the leukemia lines studied and normal bone marrow
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Chart 3. Survival as a function of duration of heating at 41°, 42°, 43°, and 45° for AKR leukemia cells heated in vitro. The effects of in vitro incubation at 37° is also shown. Different symbols within a temperature group represent different experiments. Bars, S.E.

Table 1

<table>
<thead>
<tr>
<th>Temperature °</th>
<th>NCFU D0 (min)</th>
<th>n</th>
<th>L1210 D0 (min)</th>
<th>n</th>
<th>AKR D0 (min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.0</td>
<td>68</td>
<td>2.0</td>
<td>58</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42.0</td>
<td>14</td>
<td>1.5</td>
<td>16</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43.0</td>
<td>7.6</td>
<td>1.0</td>
<td>4.0</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45.0</td>
<td>1.6</td>
<td>1.7</td>
<td>1.0</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

were not due to inherent differences between the mouse strains.

Malignant cell populations usually exhibit a significantly higher fraction of proliferating cells, as compared to normal cell populations, a majority of which usually are not cycling. To determine the importance of this difference on thermal sensitivity, we stimulated normal bone marrow cells into cell cycle by either cyclophosphamide or by transplantation of untreated bone marrow into heavily irradiated recipient mice. These procedures have been previously shown to stimulate most, if not all, stem cells into active proliferation (31).

Cyclophosphamide reduced the number of NCFU per femur to 46% (740 NCFU/femur) of normal, while bone marrow transplanted into irradiated animals yielded a NCFU pool about 15% (240 NCFU/femur) of that found in undisturbed normal bone marrow (1600 NCFU/femur). Chart 5 indicates the survival of regenerating bone marrow cells exposed to 42° as compared with undisturbed bone marrow. There is no difference in thermal sensitivity of regenerating NCFU whether induced by cyclophosph-
Counterparts, it was considered appropriate to compare normal treatment causes a significant cell cycle delay. The amount of killing for a given increased life span if the media. When the well-buffered α-MOPS medium was compared to medium changes, possibly due to uncontrolled pH alterations in Fisher's medium related to its poor buffering capacity or to differences in nutritional constituents between the 2 media. When the well-buffered α-MOPS medium was compared with Fisher's medium (Chart 6), no difference in thermal sensitivity at 42° of NCFU (Δ, △) and L1210 leukemia cells (○, ■, □). Different symbols represent different experiments. Bars, S.E.

**DISCUSSION**

Various reasons for hyperthermic cell killing have been discussed previously. Direct DNA damage (2, 33), interference with normal cellular repair mechanisms (1, 34), and changes in membrane function (13, 28) all seem to be possible causes. There is no doubt that cell sensitivity to heat also is enhanced by both poor nutrient conditions (12, 23) and acidosis (10, 33), those characteristics which are likely to yield some selective cell killing in solid tumors. However, there is no reason to expect, a priori, that selective killing of leukemia cells by heat would occur, since the conditions mentioned above are not likely to play a major role. Because leukemia cells which are systemically distributed are likely to be found in the same milieu as are their normal counterparts, it was considered appropriate to compare normal and malignant cells in vitro under similar conditions (i.e., physiological pH). Under these circumstances, the normal bone marrow progenitors (NCFU) show only moderate thermal sensitivity and may exhibit thermotolerance after 4 hr at 42°. These data are supported by the findings of Elkon and McGrath (8) for granulocyte-macrophage colony-forming units, although their assay could not preclude complications by heat effects on cell cycle progression.

Symonds et al. (27), using Fisher's medium, report a greater heat sensitivity of NCFU than is indicated in Chart 1. In our studies, the shoulder region seems to be particularly susceptible to medium changes, possibly due to uncontrolled pH alterations in Fisher's medium related to its poor buffering capacity or to differences in nutritional constituents between the 2 media. When the well-buffered α-MOPS medium was compared with Fisher's medium (Chart 6), no difference in thermal sensitiv-
being the increased heat sensitivity of S-phase cells which has been reported (25, 35). Indeed, it is possible that features distinguishing cycling from noncycling cells (G0) such as rates of macromolecular synthesis or membrane alterations could intrinsically account for the increased heat sensitivity. Alternatively, a probable cause might be the shortened interval of repair before the damage is irreversibly fixed during S phase or cell division in cycling cells similar to the phenomenon of heat-induced potentially lethal damage repair (17).

The difference in thermal sensitivity between normal and malignant cells can also be seen in an Arrhenius plot (Chart 7). While both AKR and L1210 cells show similar D0 values to proliferating Chinese hamster ovary cells, normal bone marrow stem cells show significantly less thermal sensitivity, as evidenced by the downward displacement of the curve for these cells. Note that all cell types studied show an indication of a break occurring at 43° with the exception, perhaps, of NCFU. This phenomenon has been consistently reported for many cell types (6, 8, 25). In addition, a greater difference in thermal sensitivity is observed at 42° than at 43° or 45°, as noted before.

Despite reports that leukemia cells are sensitive to heat, there is a surprising lack of clinical evaluation of whole-body hyperthermia on patients with leukemia. Although the clinical use of whole-body hyperthermia has been reported by several investigators treating a cumulative total of more than 200 patients (15, 21, 22, 24), nearly all of these studies have been performed on advanced solid tumors. The results shown here lend further support to the suggestion of Symonds et al. (27) that hyperthermia may be useful for autologous transplantation of bone marrow during cancer treatment. The in vitro treatment of bone marrow transplantation cells by hyperthermia might reduce or eliminate any possible contamination of malignant cells present which were undetectable during remission when the cells were obtained.

There is little evidence of normal hematological effects by whole-body hyperthermia, with the exception of Parks (21) who noted some evidence of thrombocytopenia and leukopenia, although these may have been at least partially due to the extracorporeal-heating technique he used. Ludgate et al. (18) also reported coagulation defects associated with whole-body heating. While this may suggest that normal bone marrow may not be a treatment-limiting tissue in whole-body hyperthermia alone, whole-body heating is most likely to be combined with chemotherapy in which bone marrow response is frequently treatment limiting. The fact that heat itself is relatively nontoxic to NCFU is an encouraging finding for combined therapy; however, this relative insensitivity to heat must also be tested in the presence of chemotherapeutic drugs, a situation which is known to enhance cell killing in some instances (13). Surely, these studies suggest that heat should be thoroughly investigated as a modality in hematological cancer.

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REFERENCES


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