Differential Sensitivity of AKR Murine Leukemia and Normal Bone Marrow Cells to Hyperthermia

H. Ian Robins, Richard A. Steeves, Allen W. Clark, Patricia A. Martin, Kenneth Miller, and Warren H. Dennis

Departments of Human Oncology [H.I.R., R.A.S., P.A.M., K.M.], Anatomy [A.W.C.], and Physiology [W.H.D.], University of Wisconsin Medical School, Madison, Wisconsin 53792

ABSTRACT

To determine if there is a differential effect of hyperthermia on AKR murine leukemia and AKR normal bone marrow cells incubated in vitro, the fractional survival of leukemic and of normal cells with proliferative potential as a function of heating exposure was estimated by evaluating spleen colony formation. Normal bone marrow colony-forming units were assayed in lethally irradiated (750 centigrays) mice; leukemic colony-forming units were assayed in nonirradiated mice. Electron micrographic studies of leukemic cells treated with 41.8° hyperthermia found that structural damage to the cell, i.e., changes in the Golgi apparatus, was associated with the lack of ability to form colonies. AKR leukemia cells were more sensitive than normal cells to hyperthermic killing at 41.8° and at 42.5°. This differential was found whether cells of each type were heated separately or when mixed together. This model system demonstrates an inherently greater sensitivity of neoplastic cells, as compared to normal syngeneic stem cells, to thermal killing. This finding may have relevance to autologous bone marrow transplantation in humans.

INTRODUCTION

Reinfusion of autologous bone marrow obtained during remission of leukemia eliminates problems which arise from HLA incompatibility when one uses nonautologous cells. However, the infused marrow may contain undetected leukemic cells (8). A method of purging leukemic cells from stored bone marrows prior to transfusion is a desirable clinical aim.

There are several neoplasms which are more sensitive to hyperthermic killing than their normal cell counterparts (6, 9, 11, 12, 14). That this differential sensitivity may exist in human leukemias and that heating autologous bone marrow transplants in vitro may selectively reduce the number of neoplastic cells reinjected have been speculated (13, 17, 20).

We chose the AKR murine leukemia model for conducting preclinical in vitro studies to complement our in vivo studies of the interaction of whole-body hyperthermia and whole-body irradiation (15, 16). Parenthetically, such studies, of course, are the other side of the transplant coin, i.e., how to remove leukemic cells from the host. The AKR model is nonimmunogenic (1), and cell loss can be studied quantitatively with the spleen colony method. Further, survival of both normal hematopoietic and of transplanted leukemic colony-forming cells can be estimated (2-4).

MATERIALS AND METHODS

Mice. Female AKR mice, 6 to 7 weeks old, weighing 20 to 25 g, were obtained from Cumberland Farms (Clinton, Tenn.). 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 (2). The ascites line (provided by Dr. Rex Risser, McArdle Laboratory, University of Wisconsin-Madison) was adapted to a spleen line by i.v. injection via tail vein of a spleen perfusate with a new passage every 7 to 10 days (see below). After 10 passages, the adaptation as a splenic line met our needs.

Media. For spleen perfusion, cold (2-4°C) medium containing 10% newborn bovine serum in Eagle’s minimal essential medium (modified) with Earle’s salts and with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and without bicarbonate buffer or glutamine (Flow Laboratories, McLean, Va.) was used. Glutamine was replenished on the day of the experiment (292.3 mg/liter, Flow Laboratories). The elimination of bicarbonate buffer and the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer to the phosphate buffer present provide for a pH of 7.46 ± 0.05 (S.E.) at all temperatures and time points under the conditions of our studies.

Leukemia Cell Preparation. The spleen from a leukemic AKR mouse was removed and weighed. Spleens weighing more than 0.3 g were used for preparation of splenic perfusate. Cold medium was forced into the spleens via a 10-ml 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 (No. 2070; Falcon Plastics, Oxnard, Calif.). Cell clumps and spleen fragments were allowed to settle, the upper portion (9 ml) was removed by pipet, and the suspension was centrifuged at 2°, 1200 rpm for 10 min (RC-3; Sorval, New Town, Conn.). The supernatant was discarded, and the pellet was resuspended in 10 ml of media. An aliquot of the cell suspension was diluited 1:20 in Turks solution (2% glacial acetic acid in distilled water), and the concentration of nucleated cells was determined by a hemocytometer count. The suspension was then diluted to 10⁶ cells/ml using cold medium.

Bone Marrow Preparation. Tibiae and femora were removed from nonleukemic AKR mice. The ends of the bones were clipped, and bone marrow plugs were flushed out with medium through a 26-gauge needle. Plugs were disrupted by gently aspirating plugs up and down a 1-ml syringe equipped with a 22-gauge needle. The resulting suspension was processed as described above.

Spleen Colony Assay. The sensitivity of the assay has been demonstrated previously for both types of tissue (19). 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 were injected into each of 5 to 10 mice which had been lethally irradiated with 750 centigrays (¹³⁷Cs source; Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada). Irradiated assay mice were maintained with drinking water containing tetracycline until sacrifice. On Day 9, the animals were sacrificed; spleens were removed and placed in...
Bouin's fixative. The number of macroscopic colonies on each spleen was counted and the number of N-CFU in the initial suspension determined (4). A lethally irradiated group into which no exogenous bone marrow cells were injected was always present for estimating the frequency of endogenous colony formation. Bone marrow cells in these experiments had a colony-forming efficiency (2) of 1.5 CFU/10⁶ cells.

AKR leukemia cells were similarly assayed, but in nonirradiated mice which were sacrificed 8 days after injection. These L-CFU had a colony-forming efficiency (2) in these experiments of 100 to 150 CFU/10⁶ cells.

We find 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% Eagle's minimal essential medium). Aliquots from this stock are being used for up to 10 passages, i.e., passage numbers 20 to 30.

In Vitro Incubations. Ten ml of cells (10⁶ cells/ml) were incubated inside 50-ml Corex tubes (No. 8080-A; Corex; Corning Glassworks). Tubes were loosely capped with aluminum foil. One tube was used for each time point. Tubes were maintained in ice prior to placement in water baths. The Corex tubes have a reasonable thermal conductivity and allow for temperature equilibration within 4 min after placement in water baths.

At time zero, the tubes were placed in Precision Shaking Water Baths (American Scientific Products, Mcgraw Park, Ill.). The water was heated to appropriate temperature ±0.1° while covered with 20-mm polypropylene spheres to help maintain constant temperature. The tube rack was shaken at approximately 160 oscillations/min. Equilibration to within 0.1 between the tube contents and the water bath temperature was achieved in approximately 4 min. In addition to mechanical shaking, the tubes were gently swirled by hand every 30 min to reduce cell settling.

Water bath temperature measurements were made with a 0–50° (0.1°) thermometer (No. 15-043A; manufactured to meet National Bureau of Standards Monograph No. 90, Fischer Scientific, Pittsburgh, Pa.).

At each time point, the tubes were removed and placed on ice. To dispense any remaining cell clumps, the cell suspensions were aspirated up and down a 10 ml pipet 10 times. The nucleated cells were then counted, and an appropriate dilution was made for injection into assay mice as described above.

Mixed Cell Experiment. Unless otherwise noted, in the experiments in which leukemic and bone marrow were mixed, suspensions of 10⁶ leukemic cells/ml (either irradiated or unirradiated cells) were added to equal volumes of 10⁶ bone marrow cells producing a final concentration of 2 × 10⁶ cells/ml.

To assay for leukemic cell survival in the presence of bone marrow, suspensions of nonirradiated leukemic cells were mixed with bone marrow cells and incubated at hyperthermic temperature, and aliquots were injected into nonirradiated assay mice. With this assay, only L-CFU should be observed (1, 3, 5). To assay for bone marrow survival (N-CFU) in the presence of leukemic cells, suspensions of leukemic cells were lethally irradiated while at 2–4° (7000 centigrays), 137Cs source (Atomic Energy of Canada, Ltd.). These cells were mixed with bone marrow cells and incubated at hyperthermic temperature, and aliquots were injected into lethally irradiated assay mice (see above). In this assay, only N-CFU should be observed (3, 5, 19).

Electron Microscopic Studies. Samples of leukemic cells were incubated at 41.8° for 1 hr as described above and then centrifuged at 2, 1200 rpm for 10 min (RC-3; Sorval). The supernatant was discarded, and the pellet was fixed with 4% glutaraldehyde: 2% formaldehyde: 0.1 M phosphate buffer, pH 7.4, at room temperature (21°) for a total time of 1 hr. The fixed pellets were thoroughly rinsed in plain buffer and then placed in 1% OsO₄, 0.1 M phosphate buffer, pH 7.4, for 1 hr. The pellets were stained for 2 hr in aqueous uranyl acetate (11) and then embedded according to standard methods. For electron microscopy, pellets were sectioned with diamond knives, stained with uranyl acetate and lead citrate, and photographed on either a Philips EM410 or a JEOL 100 CX electron microscope.

Alternatively, samples incubated at 41.8° for 1 hr were transferred to a second water bath set at 37° and heated for 1 additional hr. Following this second incubation, cells were fixed as described above. In addition, cells for comparison were incubated at 37° for 2 hr and then fixed and stained.

RESULTS

The colony-forming ability of leukemic and normal bone marrow cells as a function of time of in vitro incubation is illustrated in Chart 1 for 41.8° and in Chart 2 for 42.5°. At both temperatures, there was an exponential decrease in leukemic and normal CFU with time. At 41.8°, no shoulder is seen in the survival curves. In contrast, the survival curve for 42.5° incubation shows a shoulder for both normal and leukemic cells. In both Charts 1 and 2, the terminal slopes of the curves demonstrate greater...
Survival of each cell type was not affected by the presence of the other cell type. In the experiment, the 10^6/ml bone marrow cells were heated alone (O) or in the presence of 10^6 normal bone marrow cells/ml. In the presence of normal cells, no difference in survival is observed. Similarly, the survival data are shown in the upper curve (Chart 3) for normal bone marrow cells with and without 10^6 lethally irradiated leukemic cells/ml. Again, the presence of the second cell type had no effect on hyperthermic sensitivity.

The electron microscopic photographs of cells fixed after incubation at 37° for 1 to 2 hr had no detectable, notable morphological changes (Fig. 1). However, leukemic cells incubated at 41.8° for 1 hr gave images showing changes in the Golgi apparatus in most cells. Instead of the normal array of flattened sacs (Fig. 1), the Golgi apparatus of these cells was vesiculated or swollen (Fig. 2). In many instances, identification of the apparatus was difficult. (Our impression was that such changes were uncommon in normal cells incubated at 41.8°; however, quantitation of this impression was not attempted.) In leukemic cells which were incubated at 41.8° for 1 hr followed by a second incubation at 37° for 1 hr and then fixed, significant numbers of cells demonstrated further morphological changes consisting of the breakdown of the nuclear envelope, clumping of cytoplasmic organelles, as well as extraction of the cytoplasm and nucleoplasm and breakdown of the plasmalemma in many cases (Fig. 3).

DISCUSSION

Kase and Hahn (12) review, in part, examples of the differential sensitivity of normal versus neoplastic cells of the same origin. The present study involving a syngeneic cell line adds another example to a limited literature. In this study, we have compared the hyperthermic sensitivity of a T-cell neoplasia that originates in the thymus and disseminates to bone marrow with the heat sensitivity of normal bone marrow hematopoietic cells. Symonds et al. (18) have studied a comparable syngeneic example (L1210 cells grown in DBA/2 mice). They, using methods comparable to those used here, report that, in the simultaneous heating of normal marrow cells with L1210 cells, at 43°, the presence of the second cell type had no effect on hyperthermic sensitivity.

The electron microscopic studies presented here further support the concept that the observed decrease in CFU is secondary to leukemic cell death. The changes seen in the Golgi apparatus (Fig. 1) were also seen in a previous hyperthermia study involving CA755 mammary adenocarcinoma in C57BL × DBA/2 F1 mice (7). In that study, similar Golgi apparatus changes correlated with tumor cell death, with increase in survival and with cures in some animals. [Both the CA775 model as well as the AKR model are poorly immunogenic (1, 10).] Additionally, the morphological changes which occur in AKR leukemia cells heated for 41.8° for 1 hr followed by a second incubation for 1 hr at 37° (Fig. 3) also suggest that the 41.8° incubation may result in cell death. The second incubation is necessary to permit morphological changes which are correlated with cell death to be fully expressed.

We believe that the decreased ability of heated AKR leukemic cells to form colonies represents cell death. This conclusion is supported by recent in vivo experiments in which leukemic AKR mice received 41.8° whole-body hyperthermia, and the effect of hyperthermia was determined after splenectomy by assessing growth of L-CFU as well as survival. We consistently found that a decrease in CFU was associated with increased survival. Since a decrease in L-CFU at 41.8° in vivo is due to leukemic cell death, one can extrapolate that the decrease in L-CFU at 41.8° in vitro is similarly related.

The electron microscopic studies presented here further support the concept that the observed decrease in CFU is secondary to leukemic cell death. The changes seen in the Golgi apparatus (Fig. 1) were also seen in a previous hyperthermia study involving CA755 mammary adenocarcinoma in C57BL × DBA/2 F1 mice (7). In that study, similar Golgi apparatus changes correlated with tumor cell death, with increase in survival and with cures in some animals. [Both the CA775 model as well as the AKR model are poorly immunogenic (1, 10).] Additionally, the morphological changes which occur in AKR leukemia cells heated for 41.8° for 1 hr followed by a second incubation for 1 hr at 37° (Fig. 3) also suggest that the 41.8° incubation may result in cell death. The second incubation is necessary to permit morphological changes which are correlated with cell death to be fully expressed.

We believe that our results support the argument that in vitro hyperthermia may serve as a useful adjunct to clinical bone marrow transplantation in the treatment of acute leukemia. Further exploration of this concept using human tissue in vitro is, therefore, encouraged.

ACKNOWLEDGMENTS

We wish to express our gratitude to Linda M. Shecterle for outstanding technical and editorial assistance.

REFERENCES

9. Giovannelli, B. D., Morgan, A. C., Stehlin, J. S., and Williams, L. J. Selective

Chart 3. Surviving fractions calculated as in Chart 1. Heating was at 41.8°. In the experiment, the 10^6/ml bone marrow cells were heated alone (O) or in the presence of 10^6 lethally irradiated AKR leukemia cells (A). The AKR leukemia cells (10^6/ml) were heated alone (A) or in presence of bone marrow cells (10^6/ml) (A). Survival of each cell type was not affected by the presence of the other cell type.

OCTOBER 1983

4953

Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 1983 American Association for Cancer Research.


Fig. 1. Electron micrograph of a portion of an AKR lymphoma cell incubated at 37° for 2 hr before fixation. At the cytocenter, a pair of centrioles (C), several arrays of the Golgi apparatus (G), mitochondria (m), elements of the endoplasmic reticulum (arrow), free polyribosomes, and a portion of the nucleus (N) are visible. x 47,600.

Fig. 2. Electron micrograph of a portion of an AKR lymphoma cell incubated at 41.8° for 1 hr before fixation. All of the elements noted in Fig. 1 are visible here and have the same morphology except the Golgi apparatus. x 47,600.

Fig. 3. Electron micrograph of a portion of an AKR lymphoma cell incubated at 41.8° for 1 hr and then at 37° for 1 hr before fixation. The nuclear envelope in this cell is no longer visible so the chromatin (c) appears to be in direct contact with the cytoplasm. Mitochondria (m) also are visible, as are scattered elements of the endoplasmic reticulum (arrow). x 31,100.
Differential Sensitivity of AKR Murine Leukemia and Normal Bone Marrow Cells to Hyperthermia


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/10/4951

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.