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Studies of Nitrogen Mustard Transport by Mouse Myeloma and Hemopoietic Precursor Cells[1]

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SUMMARY

We studied the transport of nitrogen mustard (HN2) by mouse hemopoietic and transplantable myeloma (Adj. PC-5 and MOPC 460D) stem cells, using gradients of temperature and choline chloride. Bone marrow and myeloma cells were exposed to HN2 in culture at various temperatures, washed, and tested for colony-forming efficiency. The protective effect of lowered temperatures was similar for normal marrow colony-forming units and both lines of myeloma cells. When cells were exposed to HN2 in the presence of choline chloride, the survival of colony-forming units increased, and the dose response curves were similar among the three classes of cells. We were unable to demonstrate a qualitative difference in the transport of HN2 by mouse hemopoietic precursor cells and the stem cells of two myelomas with different sensitivities to HN2.

INTRODUCTION

The effectiveness of a cancer chemotherapeutic agent should be assessed on the basis of its differential effects on tumor and normal cells. We recently reported that transplantable mouse myeloma (Adj. PC-5) stem cells were more sensitive than mouse hemopoietic precursor cells to HN2[3] when survival was assessed by means of clonal cell culture techniques (5). Single-cell suspension of both myeloma and bone marrow cells were exposed to HN2 for 1 hr, washed free of the drug, and assayed for the survival of colony-forming cells. When surviving fractions of both myeloma and bone marrow CFU were plotted on a semilogarithmic scale against doses of HN2, the slopes of the survival curves showed that myeloma stem cells are 25-fold more sensitive than bone marrow precursor cells. In addition, it was found that the difference of sensitivity was not dependent on the difference in the proportions of cells in cycle; rather, it was dependent on differences in the intrinsic properties of the 2 cell classes. Subsequently, we observed that the sensitivity of another transplantable mouse myeloma MOPC 460D to HN2 was closer to that of the hemopoietic stem cells. This report concerns our attempt to delineate the mechanisms that determine the intrinsic sensitivity of each class of cells to HN2.

We attempted to identify qualitative and quantitative differences in the transport of HN2 by mouse hemopoietic and myeloma (Adj. PC-5 and MOPC 460D) stem cells, using gradients of temperature and choline chloride. Results indicate that there are no qualitative differences in the transport of HN2 between myeloma and hemopoietic stem cells. We could not determine whether the difference in the intrinsic sensitivity of these stem cells to HN2 depends on quantitative differences in drug transport or the effective intracellular drug concentrations required to kill the cells.

MATERIALS AND METHODS

Culture Assay for Mouse Myeloma Stem Cells (Myeloma CFU). BALB/c female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Myeloma lines Adj. PC-5 and MOPC 460D were obtained from Dr. Michael Potter, NIH, Bethesda, Md. Adj. PC-5 was originally obtained in ascitic form and subsequently was adapted for s.c. growth. MOPC 460D was obtained as a s.c. tumor. Transplantation of each tumor was carried out every 2 weeks with a 15-gauge needle. Tumor cell suspensions were prepared from s.c. tumor with a fine wire mesh screen (1) and were cultured by means of a slight modification of the double-agar layer method described by Park *et al.* (6) Alpha medium (9) (Flow Laboratories, Rockville, Md.) was used instead of CMRL 1066 (Connaught Medical Research Laboratory, University of Toronto) (8) and L-ascorbic acid, 1 mM, was added to the daily feeding solution (7). After 6 to 7 days of incubation, the colonies were counted with the use of an inverted microscope at 35-fold magnification.

Assay for Mouse CFU-C. Bone marrow cells were obtained from femurs of female BALB/c mice as described previously (10). Marrow cells capable of forming colonies in culture (CFU-C) were assayed by a modification of the technique of Worton *et al.* (11). α medium and horse serum were used instead of CMRL 1066 and fetal calf serum. Bovine serum albumin was omitted.

Exposure to HN2 in Culture. Details have been described in a previous report (4). Single cell suspensions of myeloma and bone marrow cells were prepared at a concentration of 1×10^6 nucleated cells per ml in α medium and were incubated with HN2 (Boots Co., Nottingham, England) for

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[3] The abbreviations used are: HN2, nitrogen mustard; CFU, colony-forming units, CFU-C, marrow CFU in culture; myeloma CFU, myeloma CFU in culture; D_{37}, dose required to reduce the surviving fraction to 37% of control.

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1 hr in 15-ml plastic tubes. Except for temperature experiments, the incubation was carried out at 37° in a water bath. In experiments testing the effects of various temperatures on the cytotoxic action of HN2, the cell suspensions were kept at each specific temperature for 15 min before and for 1 hr during exposure to the agent. Where indicated by experimental design, choline chloride (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio) was added to cell suspensions immediately before exposure to HN2. At the conclusion of drug exposure, the cells were washed twice in α medium by centrifugation at 4° and assayed for surviving myeloma or hemopoietic CFU.

RESULTS

Our objective was to determine whether the differences in HN2-sensitivity between normal hemopoietic CFU and myeloma CFU could be explained by differences in the mechanisms transporting HN2 into the 2 cell classes. Two general approaches were used: 1st, we measured sensitivity to temperature for active transport of the drug into each cell class, and 2nd, we measured the capacity of choline to protect each cell class.

Effect of Temperature. Active transport of HN2 into cells is temperature dependent; if the same transport mechanism were operative in normal hemopoietic CFU and myeloma CFU, one might anticipate that each would exhibit a similar temperature-dose response relationship. Alternatively, if the drug entered the 2 cell classes by different mechanisms, the temperature dose response relationship might also be different. Chart 1 shows the results of experiments in which marrow and myeloma cells were exposed to HN2 at various temperatures. Concentrations of HN2 were chosen that reduced the survival of each class of CFU to 5% of control at 37°, that is, for marrow cells, 3.9×10^{-7} M; for Adj. PC-5, 1.56×10^{-8} M; and for MOPC 460D, 1.71×10^{-7} M. After exposure for 1 hr at the appropriate temperature, cells were washed twice and assayed for surviving fractions of CFU. It is evident from the chart that the temperature-dose response relationship was similar for CFU-C and both lines of myeloma cells. Thus, our experiments fail to demonstrate a difference in active transport among the 3 cell classes.

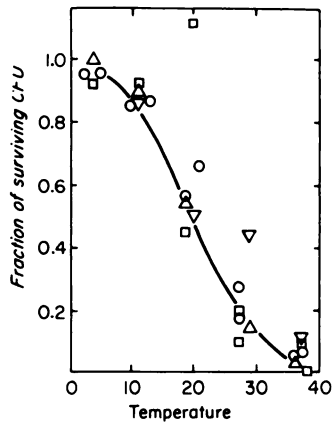

Chart 1. Sensitivity of CFU-C and myeloma CFU to HN2 at various temperatures. Marrow and myeloma cells were exposed to a concentration of HN2 that reduced the survival of CFU to 5% of control at 37°, *i.e.*, 3.9×10^{-7} M for marrow cells, 1.56×10^{-8} M for Adj. PC-5, and 1.71×10^{-7} M for MOPC 460D. At the conclusion of 1 hr of incubation with HN2, cells were washed twice and studied for the surviving fractions of CFU. O, CFU-C; Δ, Adj. PC-5; □, MOPC 460D.

Effect of Choline. Goldenberg *et al.* (2) and Lyons and Goldenberg (3) showed that choline inhibits the uptake of HN2-^{14}C by L5178Y lymphoma cells (2, 3). They interpret their finding as indicating that choline and HN2 share a common active transport mechanism. On the basis of this hypothesis, we compared the effectiveness of choline as an inhibitor of HN2 killing of normal and myeloma CFU.

In a 1st series of experiments, the 3 classes of cells were exposed to a concentration of HN2 that is known to reduce survival of colony-forming efficiency to 5% of control. The concentration is 3.9×10^{-7} M for mouse marrow cells, 1.71×10^{-7} M for MOPC 460D myeloma cells, and 1.56×10^{-8} M for Adj. PC-5 myeloma cells. α medium contains choline chloride in a concentration of 7.14×10^{-6} M, and this value is therefore the base-line concentration of choline. Increasing concentrations of choline chloride were added to the incubation mixtures of cells with drug, and the cells were washed twice and then assayed for surviving CFU (Chart 2). The dose response curves for choline protection of the 3 cell classes appear to be similar.

In a 2nd series of experiments, MOPC 460D and Adj. PC-5 were exposed to 3.9×10^{-7} M, the dose of HN2 used for normal bone marrow in the previous experimental series. The protective effect of increasing concentrations of choline chloride was then determined (Chart 3). The dashed line in Chart 3 is the response curve for normal marrow from Chart 2. A complete dose response curve was obtained for MOPC 460D. Although the curve was shifted to greater choline dose requirements because of increased sensitivity to HN2, compared to normal marrow cells, the form of the dose response curve was similar to that for normal marrow.

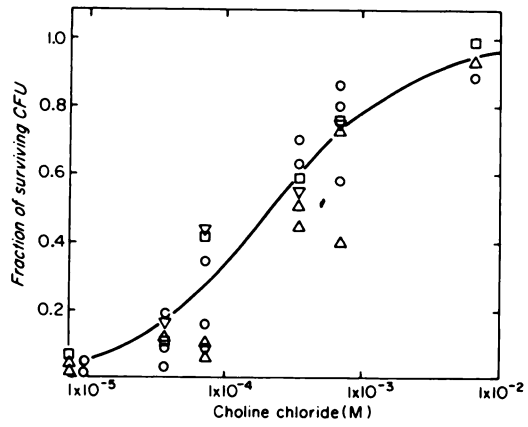

Chart 2. Effect of choline chloride on the sensitivity of CFU-C and myeloma CFU to HN2. Marrow and myeloma cells were exposed to HN2 for 1 hr in increasing concentrations of choline chloride, washed twice, and assayed for the surviving fractions of CFU. Concentrations of HN2 were chosen that reduced the survival of each class of CFU to 5% of control (which was carried out in the standard α medium containing 7.14×10^{-6} M of choline chloride), *i.e.*, HN2, 3.9×10^{-7} M for marrow cells; 1.56×10^{-8} M for Adj. PC-5; and 1.71×10^{-7} M for MOPC 460D. O, CFU-C; Δ, Adj. PC-5; □, MOPC 460D.

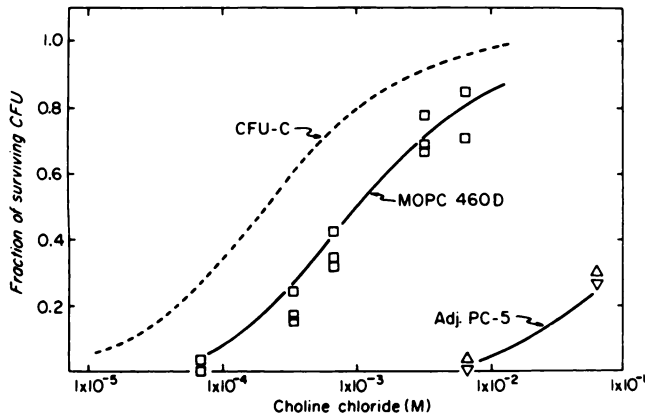

Chart 3. Effect of choline chloride on the sensitivity of CFU-C and myeloma CFU. Marrow and myeloma cells were exposed to 3.9×10^{-7} M of HN2 in increasing concentrations of choline chloride for 1 hr, washed twice, and tested for the survival of CFU. - - - -, dose response curve for CFU-C (Chart 2). Δ, Adj. PC-5; □, MOPC 460D.

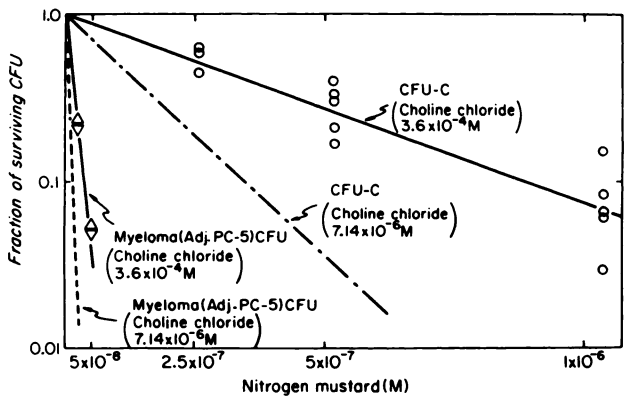

Chart 4. Sensitivity of CFU-C and myeloma CFU to HN2 in the presence of choline chloride. Marrow and myeloma (Adj. PC-5) cells were exposed to increasing concentrations of HN2 in the presence of 3.6×10^{-4} M choline chloride. The dose survival curves are plotted on a semilogarithmic scale. - - - -, survival data for the 2 classes of CFU exposed in α medium without added choline chloride and reported previously (5). O, CFU-C; Δ, Adj. PC-5.

For the highly sensitive Adj. PC-5, the killing effect of 3.9×10^{-7} M HN2 was greatly increased. Protection was achieved at 7.14×10^{-2} M choline chloride, but a complete response curve was not obtained because of toxic effect of higher concentration of choline.

In both previous sets of experiments, the protective effect of choline was tested over a limited range of HN2 doses. In a 3rd series, complete survival curves were obtained for normal marrow and Adj. PC-5 exposed to NH2 in the presence of 3.58×10^{-4} M choline chloride (Chart 4). The dashed lines, taken from previously reported experiments (5), indicate survival curves for the 2 classes of cells exposed to HN2 in α medium without added choline chloride. It is evident that choline chloride was protected over the full range of HN2 doses tested. However, the ratio between the D_{37} of the exponential survival curves for marrow CFU-C compared to myeloma CFU was similar in the presence (D_{37} of CFU-C per D_{37} of myeloma CFU = 23) and in the absence (D_{37} of CFU-C per D_{37} of myeloma CFU = 25) of additional choline chloride.

DISCUSSION

The colony-forming capacity of normal mouse granulopoietic progenitor cells and various lines of mouse myeloma cells is known to vary in sensitivity to the lethal effects of HN2 (5). The purpose of the studies reported in this paper was to seek the mechanism responsible for these differences. Since it was known that variations in the proliferative states of the various cells did not provide an explanation, a property intrinsic to the cells was examined. Specifically, we asked whether variations in transport provided the mechanism for differential sensitivity.

The nature of the cellular material did not permit a direct test of the transport hypothesis. Both normal mouse marrow and myeloma cell suspensions are heterogeneous; accordingly, a comparison of uptake of labeled HN2 into these populations would not be meaningful, since a small minority of cells is capable of colony formation. Presently available separation procedures do not provide pure populations and hence a way around this problem. Indirect measurements of uptake into cells with colony-forming capacity were not feasible; such studies are usually based on the killing of cells that take up sufficient quantities of compound labeled with high-specific activity radioisotope. HN2 labeled with sufficient specific activity was not available for such studies.

For these reasons, we used indirect methods, based on variations in the lethal effects of HN2. First, we reasoned that similar active transport mechanisms would have similar sensitivities to temperature, while different temperature sensitivities might indicate different mechanisms. In a comparison of the temperature sensitivity of the lethal effects of HN2 on normal cells and 2 lines of myeloma cells, no differences were observed.

Second, we used choline chloride, since evidence was available (2, 3) that HN2 used a transport mechanism in common with this physiological compound. Choline chloride was found to protect normal hemopoietic progenitors and both lines of myeloma cells from HN2 killing; however, the form of the dose response curve for choline protection was similar for all 3 lines, although the concentration of choline chloride required for protection varied with the HN2 sensitivity of each line.

The results reported in this paper support the view that HN2 is transported by the active temperature-sensitive system utilized by choline (2, 3). The transport of HN2 and, hence, the survival of both marrow and myeloma cells are greatly influenced by temperature and choline concentrations. No apparent qualitative difference was detected between the transport mechanism of hemopoietic progenitors and myeloma stem cells with markedly different sensitivities to HN2. However, marked quantitative differences were observed in the concentration of choline required to protect the 3 classes of cells. If the effective intracellular concentration of HN2 required to kill the 3 classes of cells is similar, these differences could be explained by more active

drug transport by the more sensitive myeloma cells. On the other hand, the differences could also be explained if there were intrinsic intracellular factors that rendered the myeloma cells sensitive to smaller intracellular concentrations of HN2. The observation that choline protects both myeloma and marrow cells to the same extent (Chart 4) appears to negate the possibility that the therapeutic index of HN2 may be increased by the concomitant use of choline.

ACKNOWLEDGMENTS

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