Protection of L5178Y Lymphoblasts by Choline and Ethanolamine against Cytocidal Effect of Nitrogen Mustard in Vitro

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SUMMARY

Structural analogs of nitrogen mustard including choline and ethanolamine, previously shown to act as competitive inhibitors of nitrogen mustard (HN2) transport, have been examined for their ability to protect L5178Y lymphoblasts against the cytocidal action of HN2 in vitro. The D0 (the dose of drug reducing survival to 37% of the initial cell population) for cells treated with HN2 was increased by 6.5-fold in the presence of 1 mM choline and 12.7-fold in the presence of 1 mM ethanolamine, and each of these observations was highly significant (p < 0.001). Protection against HN2 was also observed with hydrolyzed HN2 and the hydrolyzed monofunctional compound, hydrolyzed dimethyl 2-chloroethylamine. The time interval between HN2 treatment and addition of protective agent was critical in that one-half the protective effect was lost if the agent was added 30 min after the drug and little protection remained at 2 hr. Exposure of cells to choline prior to HN2 treatment was no more effective than administration of choline simultaneously with drug therapy. No protection was noted in cells preloaded with choline for 20 hr and then washed immediately before HN2 treatment. These findings suggested that the mechanism of protection probably depends upon direct competition between protective agent and drug for transport into the cell. Attempts to protect mice against lethal doses of HN2 and rats from the myelosuppressive effects of drug were unsuccessful.

INTRODUCTION

Sensitivity to the alkylating agent HN2 is determined in part by the ability of the tumor cell to transport drug (7, 13, 16). Previous studies have established that HN2 is transported into L5178Y lymphoblasts by an active carrier-mediated process (6, 7). The carrier involved has been identified as the transport carrier for choline, a close structural analog of HN2 (6). In 1948, Barron et al. (1) recognized the structural similarity between HN2 transformation products and choline and demonstrated that choline protected rabbit bone marrow slices in vitro against the inhibitory effect of HN2 on respiration (2). In this study several structural analogs of HN2 including choline and ethanolamine, previously shown to act as competitive inhibitors of HN2-transport, have been examined for their ability to protect cells against the cytocidal action of HN2. Such a finding might provide opportunities to increase the therapeutic index of HN2 by administration of the protective agent in tandem with the active drug. This approach has been exploited to produce more effective therapy of leukemia and cancer with the use of the antimetabolite methotrexate and its specific antagonist folic acid (8, 9, 11, 14).

MATERIALS AND METHODS

Murine leukemia L5178Y lymphoblasts with a doubling time of 10 to 11 hr were grown in cell culture as previously described (4-7). Exponential phase cells at a concentration of 10^6 cells/ml in Fischer medium (Grand Island Biological Company, Grand Island, N. Y.) were treated with HN2, 0.01 to 0.05 µg/ml (0.05 to 0.25 µM) for 5 hr at 37°C in the presence and absence of choline, ethanolamine, HN2-OH, or HN1-OH at a concentration of 0.1 or 1 mM. HN2 and HN1 were hydrolyzed in 0.1 N NaOH at 60°C for 2 hr, the pH was adjusted to 7.5, and the solution was filtered through a Millipore membrane as reported previously (7). The treated cells were washed once with 5 ml of medium, and dose-survival curves were determined by the cloning method of Chu and Fischer (3). Linear regression analysis of the dose-survival curves was performed by the cloning method of Chu and Fischer (3). Linear regression analysis of the dose-survival curves was performed, the regression equations being in the form log y = mx + b, where y is surviving cell fraction, x is dose of HN2 in µg/ml, m is slope of regression line, and b is the y intercept. D0 (the dose of drug reducing survival to 1/e, i.e., 37% of the initial cell population) was derived from the negative reciprocal of the regression line.

An investigation was undertaken to determine the ability of choline to protect rats from the hemopoietic toxicity of HN2. Wistar albino male rats (Canadian Breeding Laboratories, St. Constant, LaPrairie City, Quebec, Canada) weighing 225 to 300 g were given i.p. injections of HN2, 1.75 mg/kg (9.06 µmoles/kg), immediately after establishing an i.v. infusion of 0.9% NaCl solution or choline in a femoral vein. A stock solution of choline containing 18
mg/ml was administered at a schedule of 450 μg per g body weight per 25 μl so that the final dose of choline was 450 mg/kg (3.22 mmoles/kg); the dose of choline approached the 50% lethal dose range of 590 to 750 mg/kg reported for rats given i.p. injections of a choline solution of 20 mg/ml (10). Control rats were infused with 25 μl of 0.9% NaCl solution per g body weight. Infusion rates were controlled with a Sigmamotor peristaltic pump (Sigmamotor Co., Middleport, N. Y.), the total volume of 5.5 to 7.5 ml of 0.9% NaCl solution or choline being administered in 2.5 to 3.5 hr, so that the rate of choline administration was approximately 150 mg/kg/hr. Seven pair of rats, matched for weight, were treated and daily white blood cell and platelet counts were determined on tail vein blood; the data were analyzed by a 2-tailed t test comparing the significance of the difference of the means.

**RESULTS**

Dose-survival curves of L5178Y lymphoblasts treated with HN2 in the presence and absence of choline are shown in Chart 1. The D0 for cells treated in the presence of 1 mM choline was 60.64 ng/ml and that for control cells was 9.36 ng/ml; thus cells treated in the presence of choline were 6.5-fold more resistant to HN2 and the difference was statistically significant (p < 0.001). The dose range of HN2 was 0.05 to 0.25 μM so that the molar ratio of choline to HN2 varied from 4000:1 to 20,000:1.

A similar experiment was performed with ethanolamine acting as protective agent (Chart 2). The D0 for L5178Y lymphoblasts treated with HN2 in the presence of 1 mM ethanolamine was 86.3 ng/ml and that of control cells was 6.8 ng/ml. Therefore cells treated in the presence of ethanolamine were 12.7-fold more resistant to drug and the difference was highly significant (p < 0.001). The molar ratio of ethanolamine to HN2 also ranged from 4,000:1 to 20,000:1.

The protective action of choline, ethanolamine, and 2 other structural analogs of HN2, the hydrolyzed derivative HN2-OH and the hydrolyzed monofunctional compound HN1-OH, each at a concentration of 0.1 mM was determined as shown in Table I. The D0, in the presence and absence of protective agent, was obtained from dose-survival curves similar to those shown in Charts 1 and 2. The degree of protection was expressed as the ratio of D0 in the presence of protective agent to that observed in the unprotected control; for each of the 4 compounds tested cell survival was 1.5- to 2.36-fold greater in the presence of protective agent. The protective effect of 0.1 mM choline and ethanolamine was 4- and 6-fold lower, respectively, than that observed with a 1 mM concentration of each agent (Charts 1 and 2).

The influence of the time interval between HN2 treatment and addition of protective agent is illustrated in Chart 3. For both choline and ethanolamine, a prompt decline in protection was noted if the agent was added after HN2 treatment. Approximately one-half of the protective effect was lost if the agent was added 30 min after HN2 and little
protection remained with a 2-hr interval. The addition of 1 mM choline to cell cultures 1 hr prior to HN2 treatment was no more effective (D0, 7.41 ng/ml) than administration of choline simultaneously with drug therapy (D0, 7.93 ng/ml). Cells exposed to 1 mM choline for 20 hr, and then washed, resuspended in regular medium, and treated with HN2 showed similar sensitivity (D0, 7.93 ng/ml), to cells not exposed to choline (D0, 8.38 ng/ml).

In several in vivo experiments, an attempt was made to rescue DBA/2 female mice (The Jackson Laboratories, Bar Harbor, Maine) challenged i.p. with HN2, 6.2 mg/kg (0.032 mmole/kg), a lethal dose of drug (15), by the simultaneous i.p. or s.c. administration of choline. It was necessary to limit the dose of choline since doses of 2.5 mmole/kg and greater consistently produced acute toxicity characterized by convulsions, respiratory distress, and death within minutes. Two groups of 5 female DBA/2 mice each were given i.p. injections of 0.032 mmole/kg HN2 alone or in combination with choline, 1 mmole/kg. Both groups of mice died within 6 to 12 days confirming previous reports of HN2 toxicity (15) and indicating that a molar ratio of choline to HN2 of 30:1 failed to protect mice against a lethal challenge of drug.

The white blood cell and platelet counts of rats treated with i.p. HN2 and a simultaneous i.v. infusion of either 0.9% NaCl solution or choline, the latter at a concentration 355-fold greater than that of drug, were remarkably similar. The nadir for WBC counts appeared at 3 to 4 days in both groups and only the difference in mean WBC noted at 3 days was statistically significant (p < 0.02); little effect was noted on platelets, only the counts on day 1 differed significantly (p < 0.01). These minor differences would not appear to have any biological significance particularly since they were not associated with changes in survival. Deaths were noted in 2 of 7 rats infused with 0.9% NaCl solution on Days 5 and 7 and in 5 of 7 rats infused with choline, 2 on Day 5 and 1 each on Days 6, 8, and 9.

**DISCUSSION**

The D0 for exponentially dividing L5178Y lymphoblasts treated in vitro with HN2 showed a 6- to 12-fold increase in the presence of choline or ethanolamine, but this required a 4,000 to 20,000-fold excess of either compound. This finding is particularly striking since the transport Km for choline was 25 μM and that for HN2 was 135 μM indicating that the binding affinity is about 5 times greater for choline (6). Furthermore, the concentration of HN2 in these studies was approximately 500 to 2,500 times lower than the Km for HN2 influx, whereas the level of choline was 4 to 40 times greater than the Km for choline transport. Despite these conditions, which overwhelmingly favor choline transport, a log-fold reduction in choline concentration resulted in a sharp reduction in protection (Table 1). The requirement of a great excess of choline suggests several possibilities: (a) that transport may be a relatively minor factor in the overall cytotoxic action of HN2; (b) that the rate of metabolism of
choline and other protective agents may be rapid relative to that of HN2 even though alkylating agents generally are reactive compounds, the $t_{1/2}$ of HN2 in Fischer medium being 75 min (4); and (c) that the semilogarithmic nature of dose-survival curves tends to minimize the protective effect.

For example, assuming that SCF continues to be characterized by a single exponential function of HN2, then extrapolation of the dose-survival curves in Chart 2 shows that SCF of unprotected cells treated with 1 $\mu$M HN2 would be $1.08 \times 10^{-12}$ compared with a SCF of $9.5 \times 10^{-8}$ for cells treated in the presence of 1 $\mu$M ethanolamine. (These estimates of SCF were derived directly from the corresponding regression equations.) Thus, a 1000-fold excess of ethanolamine would reduce the cytocidal effect of HN2 by 10 to 11 logs, a more impressive index of protection.

Not only a log-fold reduction in the concentration of protective agent (Table 1) but also delay in addition of the compound until 1 hr after drug treatment (Chart 3) produced a sharp drop in protection. Exposure of cells to choline 1 hr prior to HN2 treatment was no more effective than the addition of choline simultaneously with drug. No protection was observed in cells preloaded with choline for 20 hr and then washed immediately before HN2 treatment, suggesting that intracellular choline or metabolic derivatives are not significantly involved in protection. These findings suggest that the mechanism of protection may depend upon interaction between protective agent and drug at the level of the transport carrier. This postulate is consistent with previous observations that choline and HN2 share the same transport system in L5178Y lymphoblasts (6, 7) and with the finding that other competitive inhibitors of HN2 transport such as ethanolamine, HN2-OH, and HN1-OH (5-7) also protected cells against the cytocidal effect of HN2. Interaction between HN2 and protective agents at an intracellular site has not been eliminated by these studies.

The possibility of rapid metabolism of choline leading to reduction of effective concentration was considered in the design of in vivo tests of protection. Rats were infused i.v. with choline immediately before and for approximately 3 hr after HN2 treatment. Only minor differences were noted in WBC and platelet counts in rats infused with either 0.9% NaCl solution or choline, the latter at a concentration 355-fold greater than that of HN2. Furthermore, mortality was not reduced in that deaths occurred in 2 of 7 rats treated with HN2 alone compared to 5 of 7 rats treated with HN2 and choline.

The failure to demonstrate a protective effect of choline in vivo may reflect the inability to achieve a molar ratio of choline to HN2 comparable to that obtained in vitro, despite the use of choline at near-lethal levels (10). Another explanation is that HN2 transport in rat hemopoietic cells may resemble that observed in human lymphoid cells, in which drug transport consists of at least 2 components, only 1 of which is shared with choline (12). In the presence of choline, or other structural analogs, unimpeded drug transport would be expected to occur on the choline-independent transport system. Finally, this study does not exclude the possibility that other treatment schedules, such as a more prolonged choline infusion, might be more rewarding or that choline might improve the therapeutic index of HN2 in the treatment of L5178Y leukemia in mice.

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