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# Sensitivity to Nitrogen Mustard as a Function of Transport Activity and Proliferative Rate in L5178Y Lymphoblasts[1]

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## SUMMARY

Sensitivity of L5178Y lymphoblasts to the alkylating agent nitrogen mustard (HN2) was found to be a function of the proliferative state of the cells. Dose-survival curves showed that exponential or log-phase cells were 2.6-fold more sensitive to the drug than were stationary-phase cells. Carrier-mediated transport of $^{14}$C-labeled HN2, hydrolyzed HN2, and the natural substrate choline was also a function of the proliferative state of the cells. Transport was more efficient in log-phase cells as manifested by a higher binding affinity between carrier and each of the three substrates; in addition transport capacity for hydrolyzed HN2 and choline was significantly greater in log-phase cells than resting cells. The greater sensitivity of log-phase cells to HN2 to a large extent can be accounted for by a more efficient transport mechanism. The activity of the transport system for HN2 may be dependent upon proliferative rate, transport being more active in rapidly dividing cells.

## INTRODUCTION

Sensitivity to the alkylating agent HN2[4] is determined in part by the ability of the target cell to transport the drug (7, 11, 17, 19). Previous studies provided evidence for a transport carrier of HN2 in L5178Y lymphoblasts (7, 8, 11) and demonstrated that the primary function of the carrier was to transport choline (9, 10). Cells resistant to HN2 demonstrated less efficient transport with a decreased affinity of the carrier for the drug and a reduced transport capacity (7, 8, 11). In this study, sensitivity of L5178Y lymphoblasts to HN2 is shown to vary not only with transport activity but also with proliferative rate. The activity of the HN2 transport system may be dependent upon proliferative rate.

## MATERIALS AND METHODS

Murine leukemia L5178Y lymphoblasts were grown in tissue culture as previously described (6). Exponentially dividing or log-phase cells with a doubling time of 10 to 11 hr were grown by maintaining cell concentration between $10^4$ and $10^6$ cells/ml. Stationary-phase or resting cells were obtained after cell number exceeded $10^6$ cells/ml, *i.e.*, after cell growth reached a plateau (Chart 1).

Exponential- and stationary-phase cells at a concentration of $10^5$ cells/ml in Fischer medium (Grand Island Biological Company, Grand Island, N. Y.) were treated with HN2 in 50-ml Erlenmeyer flasks for 5 hr at 37°. 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). Cloning efficiency (mean ± S.E.) was 41.5 ± 2.6% for log-phase cells and 26.4 ± 2.3% for stationary-phase cells.

Covariance analysis of the dose-survival curves for log and stationary phase cells was obtained with an IBM 360 computer. The linear regression equations were in the form $\log_e y = mx + b$, where $y$ = surviving cell fraction, $x$ = dose of HN2 in μg/ml, $m$ = slope of regression line, and $b$ = the $y$ intercept or extrapolation number, $n$, which describes the shoulder of the dose-survival curve and is of little practical value. $D_0$ (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 slope of the regression line.

Transport studies were performed on log and stationary phase cells with methods previously described (11). HN2 (specific activity, 3.2 mCi/mmole) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and the hydrolyzed derivative HN2-OH was prepared by alkaline hydrolysis in 0.1 N NaOH at 60° for 2 hr. Choline-1,2-$^{14}$C chloride (specific activity, 2.0 mCi/mmole) was obtained from New England Nuclear, Boston, Mass. Linear regression analysis of the Lineweaver-Burk plots was determined: the slope represents $K_m/V_{max}$ (maximal velocity of drug uptake); the $y$ intercept is $1/V_{max}$; and the $x$ intercept is $-1/K_m$. A 2-tail $t$ test was used to compare the significance of the difference of the means for slope, $K_m$ and $V_{max}$, between log- and stationary-phase cells.

The size of log- and stationary-phase cells was determined in a Coulter Model B electronic particle counter, calibrated with giant ragweed pollen (mean cell diameter, 19.5μ) and paper mulberry spores (mean cell diameter, 12.5 μ), both of which were obtained from Coulter Diagnostics, Inc., Miami Springs, Fla.

## RESULTS

Dose-survival curves for log- and stationary-phase L5178Y lymphoblasts treated with HN2 are shown in Chart 2. The $D_0$

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[4] The abbreviations used are: HN2, nitrogen mustard; HN2-OH, hydrolyzed nitrogen mustard.

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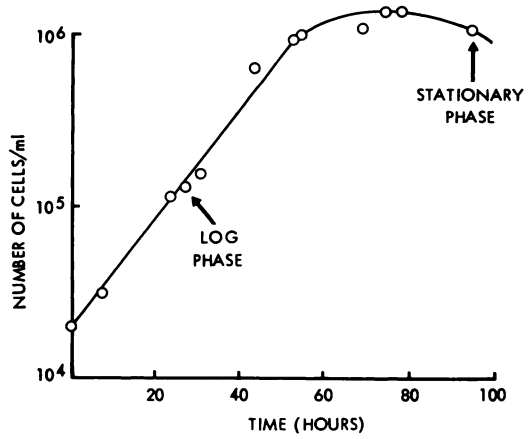


Chart 1. Growth curve of L5178Y lymphoblasts *in vitro* demonstrating exponential- or log-phase growth with a doubling time of 10 hr for approximately 50 hr followed by a resting or stationary-phase period. *Arrows*, log- and stationary-phase cells collection points for drug sensitivity and transport studies.

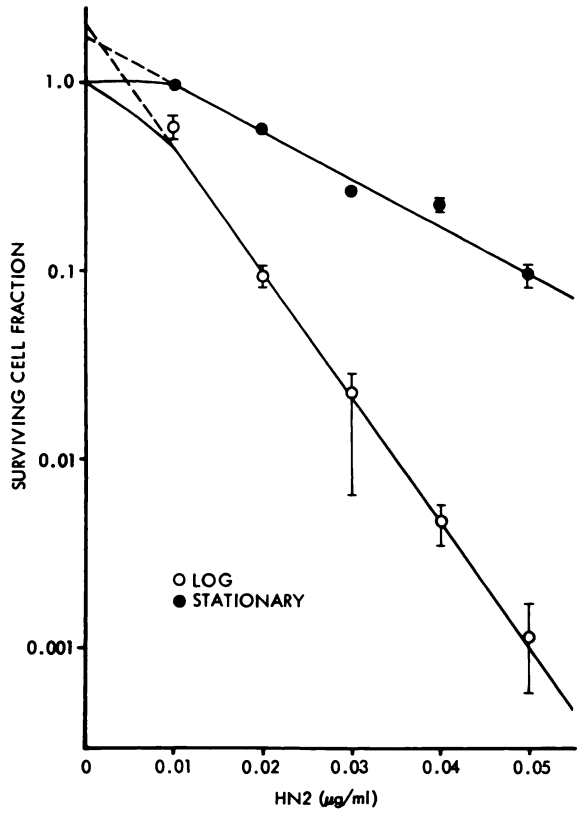


Chart 2. Dose-survival curves of log- and stationary-phase L5178Y lymphoblasts treated *in vitro* with HN2 for 5 hr and cloned in soft agar by the method of Chu and Fischer (3). *Broken lines*, an extrapolation of the regression lines to the $y$ intercept to give the extrapolation number, $n$. The regression equation for log-phase cells was $\log_e y = 153.4210x + 0.7475$ with a correlation coefficient of $-0.9769$; for stationary-phase cells the regression equation was $\log_e y = 58.2542x + 0.5662$ with a correlation coefficient of $-0.9635$. The confidence limits shown are the standard error of the mean of 4 determinations at each point.

for log-phase cells was 6.52 ng/ml, and that for stationary-phase cells was 17.17 ng/ml; thus log-phase cells were 2.6-fold more sensitive to HN2 than resting cells, and the difference was highly significant ($p < 0.001$). The $D_0$ obtained for log-phase cells with the cloning method in this study was within 8% of that previously reported with the extrapolation technique (5). Chu and Fischer (3) also reported a close correlation between the cloning and outgrowth methods. The extrapolation number ($n$) for log-phase cells was 2.11 and that of stationary-phase cells was 1.76, but the difference was not statistically significant.

Transport of all 3 substrates, HN2-$^{14}$C, HN2-OH-$^{14}$C, and choline-$^{14}$C by log- and stationary-phase cells obeyed simple Michaelis-Menten kinetics as previously described (9–11). The kinetic parameters obtained from the Lineweaver-Burk plots are shown in Table 1. The Michaelis constant, $K_m$, which represents drug concentration at half-maximal velocity is an index of the affinity of the transport carrier for drug, and $V_{max}$, the maximal velocity of drug uptake, represents transport capacity and depends on the number and mobility of transport sites. For each of the 3 substrates, HN2, HN2-OH, and choline, the mean slope of the Lineweaver-Burk plots was 2- to 3-fold higher in stationary-phase cells than in log cells, and in each case the difference was highly significant. This increase in slope signifies either an increase of $K_m$ and/or a decrease of $V_{max}$ in stationary-phase cells. With HN2 as substrate, stationary-phase cells showed an increase in $K_m$ without a significant change in $V_{max}$. Stationary-phase cells in this study showed both a higher $K_m$ and lower $V_{max}$ when compared with a more complete evaluation of log-phase cells reported previously (11). However, in order to examine transport without the complications of alkylation reactions due to HN2 (10), a kinetic analysis of HN2-OH and choline uptake by log- and stationary-phase cells was undertaken. An additional advantage of studying choline transport is that

Table 1

*Comparison of transport kinetics in log- and stationary-phase L5178Y lymphoblasts*[a]

| Proliferative state | Slope | $K_m$ (× $10^{-5}$ M) | $V_{max}$ (× $10^{-17}$/mole) min/cell) |
|---|---|---|---|
| HN2 | | | |
| Log (9) | 2.69 ± 0.14 | 10.25 ± 0.82 | 3.80 ± 0.20 |
| Stationary (3) | 5.17 ± 1.06 | 20.52 ± 3.91 | 3.97 ± 0.23 |
| $p$ | <0.01 | <0.01 | N.S.[b] |
| HN2–OH | | | |
| Log (16) | 1.58 ± 0.12 | 6.56 ± 0.55 | 4.45 ± 0.41 |
| Stationary (6) | 4.97 ± 0.65 | 8.20 ± 0.57 | 1.78 ± 0.18 |
| $p$ | <0.001 | N.S. | <0.01 |
| Choline | | | |
| Log (12) | 0.91 ± 0.08 | 2.55 ± 0.23 | 2.90 ± 0.21 |
| Stationary (6) | 2.14 ± 0.16 | 3.89 ± 0.23 | 1.85 ± 0.12 |
| $p$ | <0.001 | <0.01 | <0.01 |

[a] The data were derived from linear regression equations of Lineweaver-Burk plots of drug uptake as outlined in the text and previous publications (10, 11). The data presented are the mean ± S.E. and were statistically evaluated by the 2-tail $t$ test. The number of transport experiments performed in each group is shown in parentheses.
[b] N.S., not significant.

choline serves as the naturally occurring substrate for the HN2 transport carrier system (9, 10).

With HN2-OH acting as substrate, stationary-phase cells showed both an elevation of $K_m$ and a depression of $V_{max}$, but only the latter change reached statistical significance (Table 1). Transport of the native substrate choline by stationary-phase cells showed an increase in $K_m$ and a decrease in $V_{max}$, and both changes were statistically significant ($p < 0.01$); the increase in $K_m$ implies a reduction in the affinity of carrier for substrate, and the lower $V_{max}$ suggests a reduction of transport capacity.

An evaluation of $V_{max}$ must, however, include a consideration of cell size since $V_{max}$ is determined in part by the number of transport carriers. The mean cell volume of log cells was $1273 \pm 28$ cu $\mu$, and that of stationary-phase cells was $911 \pm 36$ cu $\mu$; the modal cell volumes of log- and stationary-phase cells were $1100 \pm 37$ and $718 \pm 36$ cu $\mu$, respectively; thus stationary-phase cells were approximately 30% smaller than exponentially dividing cells. Without knowledge of the number or distribution of transport sites on a cell membrane, the influence of cell size cannot be assessed adequately. If one assumes the same density or distribution of carriers on the membranes of log- and stationary-phase cells, then the lower $V_{max}$ in resting cells could be explained by a smaller number of transport sites on the smaller cell. However, if the number of carriers was the same on both cells, then the lower $V_{max}$ in stationary-phase cells would imply a slower carrier mobility. Attempts to relate $V_{max}$ to cell volume or surface area are futile since they do not provide the critical information on the density or distribution of transport sites. Thus the lower $V_{max}$ in stationary-phase cells may indicate either fewer transport sites and/or slower carrier mobility.

For both log- and stationary-phase cells, the $K_m$ varied with the 3 substrates, being highest for HN2, intermediate for HN2-OH, and lowest with choline, suggesting that the latter is the preferred transport substrate as previously reported (10).

Log-phase cells with a higher proliferative rate than resting cells were not only more sensitive to HN2 but also more efficient in transporting drug and native substrate as indicated by a higher binding affinity (lower $K_m$) and greater transport capacity (higher $V_{max}$).

## DISCUSSION

A classification of chemotherapeutic agents had been proposed by Bruce *et al.* (1, 2) based on the nature of the dose-survival curve obtained and the relative sensitivity of normal hematopoietic and lymphoma cells *in vivo*. Three classes of agents have been described. The 1st class, including HN2 and $\gamma$-radiation, were nonspecific agents, since they killed dividing and resting cells with comparable ease, and sensitivity was not a function of the proliferative state of the cells. The 2nd class, including thymidine-$^3$H, amethopterin, azaserine, and vinblastine, were termed phase specific, since cells were killed in only one portion of the generation cycle, and lymphoma cells with a high proliferative rate were more sensitive than normal hematopoietic stem cells. The 3rd class, consisting of 5-fluorouracil, actinomycin D, and cyclophosphamide, was termed cycle specific, because these drugs preferentially killed cells in the proliferative cycle, their action being strongly dependent on the fraction of cells in the proliferative state.

Bruce (1) has suggested that the differential sensitivity of exponential- and stationary-phase L-cells in tissue culture parallels the differential sensitivity of lymphoma and normal hematopoietic colony-forming cells *in vivo*. Exponential- or log-phase cells have a higher proliferative fraction than stationary or resting cells. Madoc-Jones and Bruce (13) using radioautography found that 45% of log-phase cells but no stationary cells incorporated thymidine-$^3$H. Hryniuk *et al.* (12) reported that the deoxynucleoside incorporation rate of log-phase L5178Y cells was 3.75- to 5-fold greater than that of resting cells.

In this study, rapidly proliferating log-phase cells with a doubling time of 10 hr were 2.6-fold more sensitive to HN2 than were stationary cells. This difference in sensitivity is similar to the report of Madoc-Jones and Bruce (13) in which exponential-phase L-cells treated with the cycle-active agent 5-fluorouracil were 3-fold more sensitive than stationary-phase cells. According to the classification of agents of Bruce, log- and stationary-phase cells should demonstrate comparable sensitivity to HN2, but in this study differential sensitivity usually associated with a cycle-active agent was observed.

Further evidence that the classification of Bruce may not have universal application is the report of Pittillo *et al.* (15) that *Escherichia coli* B in log phase was 6-fold more sensitive to HN2 than resting cultures. Another possible exception to the generalization regarding nonspecific agents is the report by Elkind and Sutton (4) that log-phase Chinese hamster cells were slightly more radiosensitive than lag-phase cells.

In this study, sensitivity to HN2 was dependent not only upon proliferative rate but also on transport activity. Kinetic analysis revealed a decreased affinity of the transport carrier for HN2 and choline and a smaller transport capacity in resting cells than in rapidly dividing cells. In a previous study (11) from this laboratory, log-phase cells made highly resistant to HN2 by repeated exposure to the drug also demonstrated a reduction in binding affinity and transport capacity. Other reports have also noted a correlation between drug uptake and sensitivity to HN2 (7, 17, 19).

Sensitivity of L5178Y cells to HN2 was a function of both proliferative rate and transport activity. As to the relationship between transport activity and proliferative rate, States and Segal (18) demonstrated that transport of the amino acid cystine was 4 times more rapid in developing than adult rat intestine, and Reiser *et al.* (16) reported a similar result for the transport of valine. In an evaluation of the regulation of a transport system for basic amino acids in *Neurospora crassa*, Pall (14) found a direct correlation between activity of the transport system and growth; transport activity was highest in rapidly growing cultures and lowest in starving cultures. The greater sensitivity of log-phase cells to HN2 in this study to a large extent can be accounted for by a more efficient transport mechanism in log-phase cells. This finding interpreted in the light of the above reports suggests that the activity of the transport system for HN2 may be dependent upon proliferative rate, transport being more active in rapidly dividing cells.

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