Nitrogen Mustard Sensitivity and Choline Transport in Walker 256 Carcinosarcoma Cells in Vitro

Gerald J. Goldenberg and B. K. Sinha
Departments of Medicine [G. J. G.] and Surgery [B. K. S.], University of Manitoba and the Manitoba Institute of Cell Biology, Winnipeg, Manitoba, R3E 0V9, Canada

SUMMARY

Single cell suspension cultures of Walker 256 carcinosarcoma were established in vitro in Fischer's medium supplemented with 10% horse serum. The cells grew exponentially with a doubling time of 16 to 17 hours, had a cell volume (mean ± S.E.) of 2313 ± 89 cu μm, and were cloned in soft agar with a cloning efficiency of 68.7 ± 6.3%. Sensitivity to the alkylating agent nitrogen mustard (HN2) was comparable to that observed previously with L5178Y murine lymphoblasts; the D0 (the dose of drug reducing survival to 37% of the initial cell population) was 5.35 ng/ml, and the extrapolation number, n, was 1.27. An investigation of the interaction of hydrolyzed nitrogen mustard and choline transport revealed that uptake of choline-14C obeyed simple Michaelis-Menten kinetics, proceeded “uphill” against a concentration gradient of over 30-fold, and showed a decrease in distribution ratio as choline concentration increased and choline transport was competitively inhibited by hydrolyzed HN2. The Michaelis constant, Km, for choline transport was 2.90 × 10^-5 M, the transport capacity, Vmax, was 4.83 × 10^-17 moles/min/cell, and the inhibition constant, Ki, with hydrolyzed HN2 as inhibitor, was 7.83 × 10^-6 M. These findings indicate that choline transport by Walker carcinosarcoma cells is an active carrier-mediated process and that choline and hydrolyzed HN2 compete for the same transport mechanism.

INTRODUCTION

Active, carrier-mediated transport of the alkylating agent HN2 has been reported previously in L5178Y murine lymphoblasts (4, 5) and in normal and leukemic human lymphoid cells (6). The carrier involved was identified as the transport carrier for choline, a close structural analog of HN2 (4, 6). This report describes the establishment of a cell line of rat Walker 256 carcinosarcoma in vitro and examines sensitivity of those cells to HN2 and the interaction of HN2-OH and choline transport.

1 This work was supported by a grant from the National Cancer Institute of Canada.
2 Clinical Research Associate of the National Cancer Institute of Canada.
3 The abbreviations used are: HN2, nitrogen mustard; HN2-OH, hydrolyzed nitrogen mustard; W256, Walker 256 carcinosarcoma.
4 Received June 5, 1973; accepted July 9, 1973.

MATERIALS AND METHODS

Walker 256 carcinosarcoma (obtained from Dr. A. C. Wallace, University of Western Ontario, London, Canada) was maintained by weekly i.m. inoculation of 150- to 200-g Wistar albino male rats (Canadian Breeding Laboratories, St. Constant, La Prairie City, Quebec, Canada) with 1 ml of tumor homogenate containing approximately 5 × 10^6 cells in Hanks’ balanced salt solution; 65 to 80% of the cells were viable by trypan blue dye exclusion. Approximately 7 days after transplantation the tumor was removed aseptically and homogenized in Hanks’ balanced salt solution; cell cultures were incubated at 37° in Fischer’s medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% horse serum at a final concentration of 5 × 10^3 cells/ml. Cell suspensions were diluted with medium every 24 to 48 hr to maintain cell concentration within a range of 4 × 10^4 to 4 × 10^4 cells/ml. Cell cultures were maintained for several months, and the cells grew exponentially with a doubling time of 16 to 17 hr. Attempts to culture cells in Dulbecco modified Eagle’s medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum were unsuccessful. On occasion, cells were frozen at the rate of 1°/min to −70° in equal volumes of tumor homogenate and preservative, consisting of 30% dimethyl sulfoxide in Fischer’s medium supplemented with 10% horse serum. An i.m. inoculation of 1 ml of rapidly thawed, frozen homogenate containing approximately 5 × 10^6 cells consistently produced tumors.

The size of W256 cells was determined in a Coulter Model B electronic particle counter, calibrated with giant ragweed pollen (mean cell diameter, 19.5 μm) and paper mulberry spores (mean cell diameter, 12.5 μm), both of which were obtained from Coulter Diagnostics, Inc., Miami Springs, Fla. The cell volume (mean ± S.E.) of W256 cells on 7 determinations was 2313 ± 89 cu μm.

Sensitivity to HN2 was determined by treating W256 cells, at a concentration of 1 × 10^5 cells/ml, with HN2 for 5 hr at 37°; the treated cells were washed once with 5 ml of medium, and dose-survival curves were obtained by the cloning method of Chu and Fischer (1). The cloning efficiency (mean ± S.E.) on 12 determinations was 68.7 ± 6.3%. Linear regression analysis of the dose-survival curve was performed, the regression equation being in the form log 2y = 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 de-
scribes the shoulder of the 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 W256 cells in suspension cultures with the use of choline-1,2-$^{14}$C chloride (specific activity, 2.0 mCi/mmmole) obtained from New England Nuclear, Boston, Mass., with methods previously described (5). Linear regression analysis of the Lineweaver-Burk plots was determined: the slope represents $K_m/V_{max}$, the $y$ intercept is $1/V_{max}$, and the $x$ intercept is $-1/K_m$. The inhibition constant $K_i$ was determined from the Lineweaver-Burk plots with the formula: apparent $K_m$ (with inhibitor) = $K_m[1 + (I)/K_i]$, where $K_m$ was obtained without inhibitor and $(I)$ was the concentration of inhibitor, as previously described (5). The hydrolyzed derivative HN2-OH was prepared by alkaline hydrolysis in 0.1 N NaOH at 60° for 2 hr.

RESULTS

A dose-survival curve of W256 cells treated with HN2 is shown in Chart 1. The surviving cell fraction followed a simple exponential function of HN2, and there was only a slight shoulder on the dose-survival curve. The $D_0$, the dose of drug reducing survival to 37% of the initial cell population, was 5.35 ng/ml and the extrapolation number, $n$, was 1.27.

A time course of the uptake of choline-$^{14}$C by W256 cells in vitro is shown in Chart 2. Uptake was linear for 60 min and thereafter decreased slightly; subsequent kinetic analysis of choline transport was terminated at 60 min to ensure that initial uptake velocity was being examined.

A kinetic analysis demonstrated that choline uptake followed simple Michaelis-Menten kinetics. The linear regression equation of the Lineweaver-Burk plot for choline transport in the absence of HN2-OH was $y = 0.6002x + 2.0708$ with a correlation coefficient of 0.9984, and that with HN2-OH present was $y = 1.3012x + 1.9051$ with a correlation coefficient of 0.9955. A $t$ test comparing the significance of the difference in slopes was highly significant ($p < 0.001$). The Michaelis constant $K_m$ for choline transport was 2.90 $\times$ $10^{-5}$ M and the transport capacity $V_{max}$ was 4.83 $\times$ $10^{-17}$ moles/min/cell. In the presence of 0.1 mM HN2-OH, choline transport was characterized by a 2.35-fold increase of $K_m$ to 6.83 $\times$ $10^{-5}$ M without any significant change in $V_{max}$ (5.25 $\times$ $10^{-17}$ moles/min/cell). This indicated that HN2-OH acted as a competitive inhibitor of choline transport, and the inhibition constant $K_i$ was 7.83 $\times$ $10^{-5}$ M.

Evidence that choline transport was an active process was the fact that uptake proceeded "uphill" against a concentration gradient of over 30-fold (Chart 3). Additional evidence for a carrier mechanism was the finding that the cell/medium distribution ratio decreased as choline concentration increased. The competitive nature of HN2-OH inhibition was also illustrated in this plot, in that inhibition of choline transport was overcome at higher substrate concentrations.

DISCUSSION

To our knowledge this is the first report of successful single-cell suspension culture of Walker 256 carcinosarcoma in vitro. Several properties of W256 and L5178Y murine lymphoma in vitro are compared in Table 1. Both
Comparison of properties of log-phase L5178Y murine lymphoma and rat Walker 256 carcinosarcoma cells in vitro

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Doubling time (hr)</th>
<th>Cell size (μm)</th>
<th>Cloning efficiency (%)</th>
<th>HN2 sensitivity</th>
<th>Choline transport</th>
<th>HN2-OH transport, K_m (×10^-3 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D_0 (ng/ml)</td>
<td>K_m (×10^-3 M)</td>
<td>K_i (×10^-3 M)</td>
</tr>
<tr>
<td>Walker 256</td>
<td>16-17</td>
<td>2313 ± 89</td>
<td>68.7 ± 6.3</td>
<td>5.35</td>
<td>2.90</td>
<td>2.55 ± 0.23</td>
</tr>
<tr>
<td>L5178Y</td>
<td>10-11</td>
<td>1273 ± 28</td>
<td>41.5 ± 2.6</td>
<td>7.72 ± 0.34</td>
<td>0.29 ± 0.09</td>
<td>4.83</td>
</tr>
</tbody>
</table>

* HN2-OH used as inhibitor of choline transport.
* Mean ± S.E.
* N.D., not done.
* Data for L5178Y cells were obtained from Refs. 2-4.

Recent experience with cloning of various L5178Y cells lines in soft agar has shown wide variation in cloning efficiency ranging from 3 to 75%.

Cell lines exhibit exponential growth curves; W256 cells have a slower doubling time of 16 to 17 hr and are almost twice as large, with a cell volume of 2313 ± 89 cu μm. The cloning efficiency in soft agar, with the method of Chu and Fischer (1), is at least as high for W256 cells; recent experience with L5178Y lymphoblasts has demonstrated considerable variation in cloning efficiency (unpublished observation).

Sensitivity to the alkylating agent HN2 was remarkably similar for both W256 and L5178Y cells. The surviving cell fraction varied exponentially with dose of HN2, and the dose-survival curves for both lines showed only a slight shoulder with a low extrapolation number; a comparison of D_0 showed that sensitivity of W256 cells in this study was 1.44-fold greater than that previously reported for L5178Y cells (3). Sensitivity of W256 cells in vitro is consistent with early studies of HN2, which demonstrated sensitivity of Walker carcinosarcoma in vivo (7).

The correspondence in HN2 sensitivity of W256 and L5178Y cells prompted an investigation of the interaction of HN2-OH and choline transport in W256 cells. It was previously shown that active transport of HN2 is mediated by the choline carrier in L5178Y lymphoblasts (4) and in normal and leukemic human lymphoid cells (6). Evidence that choline transport by W256 cells was an active, carrier-mediated process was that uptake followed simple Michaelis-Menten kinetics, that it proceeded "uphill" against a concentration gradient of over 30-fold, and that the cell/medium ratio decreased at higher substrate concentrations. The transport K_m for choline was similar in W256 and L5178Y cell lines, but the transport capacity V_max was 1.63-fold higher in W256 cells (Table 1). An evaluation of V_max must include a consideration of cell size, and W256 cells were 1.82-fold larger in volume. However, without the critical information on the density or distribution of transport carriers on the cell membrane, attempts to relate V_max to cell volume or surface area are futile (2). Thus the higher V_max in W256 cells may indicate either a larger number of transport sites and/or faster carrier mobility.

HN2-OH acted as a competitive inhibitor of choline transport with a similar inhibition constant, K_i, for both cell lines (Table 1). This finding suggests that, as with L5178Y (3) and human lymphoid (6) cells, HN2 transport by W256 cells is mediated at least in part by the choline carrier. Of interest is the fact that the K_m for HN2-OH transport by L5178Y cells was 7.76 × 10^-3 M, which is similar to the K_i of 7.38 × 10^-3 M for HN2-OH acting as inhibitor of choline transport by W256 cells. This similarity of K_m and K_i for a compound acting as substrate or inhibitor confirms the competitive nature of choline and HN2-OH transport and also suggests a similar binding affinity between HN2-OH and the transport carrier in W256 and L5178Y cells.

W256 carcinosarcoma cultured in vitro has provided a useful model for a study of HN2 sensitivity and the interaction of HN2-OH and choline transport. Parallel attempts to culture human carcinoma as single cell suspensions in vitro may prove more rewarding.

ACKNOWLEDGMENTS

We thank Ruby Ng for technical assistance and Sally Barker for typing the manuscript. HN2 hydrochloride (Mustargen) was kindly supplied by Dr. W. Dorian (Merck, Sharp and Dohme, Dorval, Quebec, Canada).

REFERENCES


Nitrogen Mustard Sensitivity and Choline Transport in Walker 256 Carcinosarcoma Cells \textit{in Vitro}

Gerald J. Goldenberg and B. K. Sinha


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/33/11/2584