Inhibition of Growth by 6-Hydroxydopamine in Cultured Cells of Neuronal and Nonneuronal Origin

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SUMMARY

The effect of 6-hydroxydopamine on growth and viability of cultured cells of sympathetic (neuroblastoma), glial, and fibroblast origin was examined. In contrast to previous reports, inhibition of growth was not specific to neuroblastoma cells. The degree of inhibition was correlated with permeability of cell membrane to dopamine-14C or 6-hydroxydopamine-14C and with sensitivity of the membrane energy-dependent transport mechanism. Inhibition of cell division could not be attributed to inhibition of ribonucleotide reductase, DNA polymerase, or thymidine kinase, since 10^-4 M 6-hydroxydopamine had no effect on these enzymes in extracts of cells in vitro.

INTRODUCTION

The discovery that 6-HD produces a chemical sympathectomy by selectively destroying sympathetic cells in vivo has exciting implications, although the mechanism of action is not understood (for a review, see Ref. 12). A selective degeneration of cultured cells of sympathetic origin by 6-HD has been reported (3). Recently, Prasad (15) found that 6-HD (and to a lesser extent, dopamine) inhibited growth of a cell line of sympathetic origin (mouse neuroblastoma) in culture at concentrations that had no effect on 2 nonneuronal cell lines, Chinese hamster ovary (CHO-K1) and baby hamster kidney (BHK-21). The inhibition of neuroblastoma growth in vitro was attributed to inhibition of cell division.

We reexamined the effects of 6-HD on a neuroblastoma (NEB) cell line in culture and extended the study to 7 glial lines (CHB4 and C6), as well as to the standard mouse fibroblast line (L929), to determine (a) the specificity of the toxicity of 6-HD to cells of sympathetic origin and (b) the mechanism of inhibition of cell multiplication by an examination of the effect of 6-HD on enzymes from NEB and L929 cell lines necessary for DNA synthesis.

MATERIALS AND METHODS

Chemicals and Radioisotopes. 6-HD and dopamine were purchased from Sigma Chemical Company, St. Louis, Mo.; 6-HD-14C (specific activity, 56.6 mCi/mmole), uniformly labeled ATP-14C (specific activity, 527 mCi/mmole), and thymidine-2-14C (specific activity, 9.6 mCi/mmole) were from Amersham/Searle Corp., Arlington Hts., Ill; dopamine-ethylamine-1-14C (specific activity, 9.6 mCi/mmole) was from New England Nuclear, Boston, Mass; and uniformly labeled CDP-14C (167 mCi/mmole) was from Schwarz/Mann, Orangeburg, N. Y. 2,5-Dihydroxyphenylalanine was the gift of Dr. E. M. Gal. All other chemicals were reagent grade.

Cell Lines. The derivation of continuous cultures of the various cell lines used in this study has been described in detail elsewhere. The NEB cells were derived from cell clones isolated from the spontaneous C-1300 Jackson neuroblastoma (4), and they exhibit many of the properties of neuronal cells (5, 16). Some neuroblastoma clones have been reported to be "adrenergic," as evidenced by measurable tyrosine hydroxylase activity, while others have no detectable tyrosine hydroxylase activity (2). The NEB cultures used hydroxylated tyrosine in stationary phase (0.14 nmole/mg protein/hr). No activity was detectable during log phase.

The CHB4 cells were derived from a cell clone reportedly of human astrocytoma origin (11). However, recent karyotype and isozyme analyses show the line to be of rat origin, and the astrocyte-like morphology, as well as detectable amounts of S-100 protein, indicate that the cells are glial in character (H. Herschman J. De Villis, and G. Sato, personal communication). Seed cultures for both NEB and CHB4 were obtained as gifts from Dr. G. Sato.

The C6 cells were originally isolated by Benda et al. (6) from rat glioma and were obtained through the American Type Culture Collection (Cell Repository Designation CCL 107).

L929 cells were originally isolated by Earle (8) from mouse connective tissue and were obtained through the American Type Culture Collection (Cell Repository Designation CCL 1). All cell lines have been maintained in monolayer culture in this laboratory for more than 1 year in Ham's Medium F10 (10) supplemented with 15% horse and 2.5% fetal calf sera, 100 units penicillin per ml, and 100 #/g streptomycin per ml and were buffered with 0.015 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.015 M NaHCO3, and 5% CO2 in balanced air. The cultures were maintained at 37° in a CO2 incubator with a subculture interval of 7 to 10 days, and medium was renewed 2 to 3 times a week.

For the purposes of this study, NEB, CHB4, and C6 were used as a system to represent cells derived from tumors of neural and glial origin, and L929 was used as a system to represent cells derived from normal tissue of other than nervous origin.
**Experimental Cultures.** We started experimental cultures by trypsinizing log phase cultures, suspending them in fresh medium, and inoculating 6 ml of this medium (containing 0.5 to 1.0 X 10⁶ cells) into 15 X 100-mm Falcon plastic Petri dishes. All cultures of a given cell line within an experimental set were started simultaneously with identical inocula and were incubated at 37°C in a 5% CO₂ balanced air atmosphere until treated experimentally. The mean population-doubling time in these experiments for NEB, CHB₄, C₆, and L929 was 28, 37, 27, and 23 hr, respectively.

**Treatment with 6-HD and 2,5-Dihydroxyphenylalanine.** Twenty-four to 48 hr after cells were plated, we washed them twice with 6 ml of Hanks' salt solution by carefully withdrawing and adding the solution to minimize any cell loss from the dish surface. The cells were then treated with 10, 50, 100, or 200 µg 6-HD per ml or 100 µg 2,5-dihydroxyphenylalanine per ml. The compounds were dissolved in Medium F10 with no serum or antibiotics but with 20 µg L-ascorbic acid per ml. (The ascorbic acid was used to minimize the autoxidation of 6-HD, the products of which are cytotoxic.) After incubation at 37°C for 1 hr, the cells were washed twice with 6 ml Hanks' solution, and 6 ml of fresh complete F10 growth medium were added. We harvested the cultures immediately (Day 0) and on 0.75, 1, 1.5, 2, or 3 days after treatment by washing them twice with Hanks' solution, trypsinizing them (0.25% trypsin in Hanks' solution) for 3 min at 37°C, and preparing a single-cell suspension by adding 6 ml of ice-cold, complete Medium F10 and pipetting it vigorously. The cells were enumerated in a hematocytometer, and viability was determined at the same time by a dye exclusion test (0.05% nigrosin in Hanks' salt solution). Growth inhibition was expressed as percentage difference between the number of cells in drug-treated and parallel control populations.

**Uptake Studies.** Cultures were washed twice with 6 ml Hanks' salt solution at either 0°C or 37°C as indicated. Three ml of fresh Medium F10, free of serum and antibiotics, and 50 µg of dopamine-¹⁴C or 6-HD-¹⁴C containing 50 µCi were added, and the cultures were incubated for the times indicated. When 2,4-dinitrophenol was added, cultures were incubated for 10 min prior to the addition of catechol derivatives. At the conclusion of the incubation, medium containing labeled compounds was decanted, cultures were washed twice with 6 ml Hanks' solution, and the cells were removed from the culture dish surface by trypsinization for 3 min at 37°C and suspended in 10 ml Hanks' solution.

After removal of an aliquot for determination of cell number and viability, cells were harvested on nitrocellulose filters (3-µm pore size; Millipore Corp., Bedford, Mass.). The filtrate contained no detectable radioactivity. The filters were air dried, and radioactivity was determined by liquid scintillation counting of filters suspended in toluene-based scintillator.

**In Vitro Enzyme Assays.** Ribonucleotide reductase was measured in solubilized extracts of NEB and L929 cells obtained by sonic treatment. Three days after inoculation (10⁶ cells/flask), cells were removed from the flasks by trypsinization. Cells from 10 flasks were pooled, chilled on ice, and packed by centrifugation at 1000 rpm in an International PR-2 centrifuge. The cells were washed twice with 0.9% NaCl solution and once with 0.02 M Tris chloride buffer; pH 7.0, containing 0.01 M MgCl₂, 0.002 M EDTA, and 0.002 M dithiothreitol. They were suspended in 0.5 ml of the above-described buffer, placed in ice, and treated sonically for 2 min prior to the addition of catechol derivatives. At the conclusion of the incubation, the products of which are cytotoxic. After incubation at 37°C for 30 min, we added 0.02 ml 10 N HClO₄ to each tube and placed them in a boiling water bath for 10 min to hydrolyze nucleotides to the monophosphates. The mixtures were then cooled, neutralized to pH 7.0 with 1 N KOH, and chilled on ice. Precipitated KC1O₄ was removed by centrifugation, and dCMP was separated from CMP on Baker-Flex PEI-F plates (18). DNA polymerase activity was assayed with the incubation mixture described by Margolis (13) with dATP-¹⁴C. Labeled DNA was isolated on filter paper discs (7). Thymidine kinase activity was measured according to the method of Sung (17). Tyrosine hydroxylase was assayed as described previously (9).

**RESULTS**

All cell lines (NEB, CHB₄, C₆, and L929) included in the study grew as monolayers, making it easy to monitor cytomorphology and to ensure that drug-containing solution came in contact with all of the cells immediately and
Growth Inhibition by 6-HD

Chart 2. Percentage inhibition \([(\text{number of control cells} - \text{number of 6-HD-treated cells}) \div \text{number of control cells}] \times 100\) as a function of 6-HD concentration. The mean percentages of growth inhibition of 4 to 6 samples of NEB, CHB4, C6, and L929 cells (± S.E.) 1 day after treatment are indicated.

Effects of 6-HD on NEB, CHB4, C6, and L929 Cells. Acute treatment (1 hr) with 6-HD affected the growth of the cell lines examined. However, the NEB cells at a 6-HD concentration of 200 μg/ml showed a reduction in viability (80 to 95% viable), whereas the viability of all other control and treated cell populations was 98% or greater.

Growth (proliferation of population by cell division) inhibition was observed after 6-HD treatment in all 4 lines in vitro, and the effect was dose dependent; however, the degree of inhibition and the time course for maximum inhibition and recovery from 6-HD treatment was cell line dependent (Charts 2 and 3).

Eighteen hr after 6-HD treatment, only those cell populations treated with 200 μg 6-HD per ml showed significant growth inhibition. Twenty-four hr (Day 1) after treatment, a clear differential is evident between the cell lines at 200, 100, and (to some extent) at 50 μg 6-HD per ml (Chart 2). The effective dose is lower and the degree of inhibition is greater in the NEB and CHB4 cell lines than in either C6 or L929.

Thirty-six hr after treatment, the pattern of differential response between the cell lines began to change and, by Day 3 (Chart 3), the growth inhibition curves of NEB, CHB4, and C6 (of neoplastic origin) were similar and were different from that of L929 (of nonneoplastic origin); i.e., the 3 cell lines of neoplastic origin were significantly more sensitive to 6-HD than the cell line of nonneoplastic origin.

Since 200 μg 6-HD per ml resulted not only in reduced viability for the NEB cells but also in general cytotoxicity for all cells, the experiments should be evaluated for specific effects at concentrations of 6-HD of 100 μg/ml or less.

Effects of 2,5-Dihydroxyphenylalanine. 6-HD is easily oxidized and its effects in vivo have been attributed to oxidation products, particularly its quinone (1). The effect of 2,5-dihydroxyphenylalanine was studied, since it is a related structure lacking the potential for quinone formation. Since the maximum differential effects on growth were observed at 100 μg 6-HD per ml the same concentration and experimental conditions were used for 2,5-dihydroxyphenylalanine. No
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Table 1
Temperature-dependent uptake of dopamine-14C and 6-HD-14C into cultured cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dopamine-14C (dpm/10^6 cells)</th>
<th>6-HD-14C (dpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB</td>
<td>37^a</td>
<td>37^a</td>
</tr>
<tr>
<td>CHB_4</td>
<td>369 ± 43</td>
<td>345 ± 14</td>
</tr>
<tr>
<td>C_6</td>
<td>215 ± 27</td>
<td>169 ± 10</td>
</tr>
<tr>
<td>L929</td>
<td>270 ± 37</td>
<td>174 ± 12</td>
</tr>
</tbody>
</table>

Table 2
Effect of 2,4-DNP on the uptake of 6-HD-14C into cultured cells

Preincubation for 10 min with 0.83 mM 2,4-DNP was followed by the addition of 50 μg (0.1 mM) 6-HD-14C containing 0.5 μCi, and incubation continued for 20 min, as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Cell line</th>
<th>6-HD-14C (dpm/10^6 cells)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB</td>
<td>Control</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>2,4-DNP</td>
<td>535</td>
</tr>
<tr>
<td>CHB_4</td>
<td>Control</td>
<td>396</td>
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<tr>
<td></td>
<td>2,4-DNP</td>
<td>879</td>
</tr>
<tr>
<td>C_6</td>
<td>Control</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>2,4-DNP</td>
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<tr>
<td>L929</td>
<td>Control</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>2,4-DNP</td>
<td>110</td>
</tr>
</tbody>
</table>

DISCUSSION

Two studies have dealt with the effects of 6-HD on neuroblastoma cells in culture (3, 15) and in vivo (3), and they have concluded that cytotoxicity and inhibition of growth were specific for cells of sympathetic origin.

We extended our studies to include 2 glial lines (of neoplastic cerebral origin) for comparison with cultured mouse neuroblastoma and a mouse fibroblast of nonneoplastic origin. Twenty hr after 6-HD treatment, a definite difference in effective dose and extent of inhibition of cell division was apparent. However, CHB_4 cells of glial origin were as sensitive as was the neuroblastoma line to the toxic effects of 6-HD. Glial line C_6 and mouse fibroblasts were affected only at higher doses, and their maximal degree of inhibition of growth was about one-half that found with the more sensitive cell lines. The results with C_6 and L929 were comparable to those reported for cells cultured from Chinese hamster ovary and baby hamster kidney cells (15), as well as HeLa and mouse sarcoma cells (3). However, if the experiments are evaluated 3 days after acute 6-HD treatment, the C_6 glial cells exhibit a degree of growth inhibition similar to that of both NEB and CHB_4 cells. Although these results do not alter the interpretation of these investigations, they illustrate the importance of the time at which an experiment is evaluated. One can possibly obtain quite different results. All experimental points had a parallel control culture that was in log phase growth, and the amount of drug that was taken up and the rate at which the cells responded to the drug were independent of cell growth rate. Thus, there is no correlation between growth rate and drug uptake or inhibition.

Some differences are apparent between our results and those reported by others. Prasad (15) found over 90% inhibition of growth by 6-HD (at a concentration of 10 μg/ml under similar conditions) in a mouse neuroblastoma line. Our results show 40% inhibition at 50 μg 6-HD per ml with NEB, and these results agree more closely with the data of Angeletti and Levi-Montalcini (3), who reported cytotoxicity at 25 μg/ml which became marked at 50 to 100 μg/ml. These differences may be due to differences in clonal origins of the cells. The NEB cells that we used were derived from a clone of the C-1300 mouse neuroblastoma, and those used by Angeletti and Levi-Montalcini were from C-1300 carried in vivo. The mouse neuroblastoma cells studied by Prasad may have had a different clonal origin.

that 6-HD was inhibitory to enzymes essential for DNA synthesis and cell division in extracts of NEB and L929 in log phase. Such inhibition of any of these enzyme systems could account for the effect on cell growth found in cell cultures. At concentrations of 10^-6 to 10^-4 M, 6-HD did not inhibit ribonucleotide reductase, DNA polymerase, or thymidine kinase in extracts of either NEB or L929 cells. Ribonucleotide reductase activities in the presence or absence of 6-HD were 15.9 ± 1.0 and 10.3 ± 0.8 nmoles per mg protein per 30 min for NEB and L929, respectively; DNA polymerase activity was 0.13 ± 0.02 nmoles dATP incorporated per mg per 30 min; and thymidine kinase activity was 4.04 ± 0.26 nmoles per mg per 30 min; neither was different in the presence of 10^-6 to 10^-6 M 6-HD.

Effect of 2,4-DNP on the uptake of 6-HD-14C into cultured cells

Cell line 6-HD-14C (dpm/10^6 cells) % of control

NEB Control 358 150
2,4-DNP 535

CHB_4 Control 396 222
2,4-DNP 879

C_6 Control 169 90
2,4-DNP 146

L929 Control 162 68
2,4-DNP 110

\* 2,4-DNP, 2,4-dinitrophenol.

Effect was seen on either cell growth or viability in any of the 4 lines, NEB, CHB_4, C_6, or L929, with this analog.

Uptake of 14C-Labeled Dopamine and 6-HD. Both dopamine-14C and 6-HD-14C were taken up by cells of all 4 lines, and the uptake was temperature dependent (Table 1). The cells derived from NEB took up 50% more dopamine-14C and had an uptake of both compounds comparable to that of the NEB cells.

Other cultures preincubated with 2,4-dinitrophenol to inhibit energy-dependent transport showed that cell lines NEB and CHB_4 exhibited similar sensitivity to this treatment (Table 2). Uptake of 6-HD-14C was increased in these cells, while that of C_6 and L929 was not affected or was slightly inhibited. Under these conditions, 2,4-dinitrophenol did not affect cell viability.

Effects of 6-HD on Ribonucleotide Reductase, DNA Polymerase, and Thymidine Kinase in Vitro. The toxicity of 6-HD to cells in culture has been attributed to inhibition of cell multiplication (15). We therefore examined the possibility of 4 lines, NEB, CHB_4, C_6, or L929, with this analog.
The differential effect of 6-HD on the various cell lines was correlated with their ability to take up both dopamine-14C and 6-HD-14C from the medium. While all of the cells exhibited a temperature-dependent uptake of labeled catechols, NEB and CHB4 had a significantly greater uptake. The sensitivity of the cellular membrane energy-dependent pump may be a factor in the differences found between cell lines.

The mechanism of the inhibition of cell division by 6-HD is still obscure. DNA polymerase, thymidine kinase, and ribonucleotide reductase, assayed in extracts of both NEB and L929 cells, were unaffected by 10^{-4} M 6-HD, suggesting that these enzymes are not the target of action of 6-HD in cultured cells. The lack of growth inhibition in cell cultures by 2,5-dihydroxyphenylalanine, an analog incapable of quinone formation, lends support to the hypothesis that the quinone oxidation product of 6-HD may be responsible for its toxic effects in vivo (1, 12).

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REFERENCES

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