Drug-induced Stimulation of Transport of Hydrolyzed Nitrogen Mustard and Choline by Normal and Leukemic Human Cells in Vitro

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SUMMARY

The effect of morphine and cocaine on the transport of hydrolyzed nitrogen mustard (HN2-OH) and choline by peripheral blood cells of normal subjects and patients with chronic lymphocytic leukemia, acute lymphoblastic leukemia, and acute myeloblastic leukemia was determined. Transport of HN2-OH by lymphocytes from normal individuals and patients with chronic lymphocytic leukemia was stimulated by morphine and cocaine and, in each case, the effect was statistically significant (p < 0.05 or greater). However, choline transport by normal lymphocytes was not altered by cocaine and was only slightly stimulated by morphine; choline transport by lymphocytes from patients with chronic lymphocytic leukemia was not stimulated by either morphine or cocaine.

HN2-OH and choline transport by cells from patients with either acute lymphoblastic or myeloblastic leukemia was stimulated to a comparable degree by both drugs. Stimulation of HN2-OH transport by morphine and cocaine was greater in normal lymphocytes than in acute leukemic cells and the differences were highly significant (p < 0.001). Conversely, stimulation of choline transport was more marked in acute leukemic cells than in normal lymphocytes, and these differences were also highly significant (p < 0.001). It was previously shown that transport of nitrogen mustard by normal and leukemic human cells was biphasic in nature, consisting of a choline-independent component at "high" drug concentrations and a choline-dependent system at "low" substrate concentrations. The preferential stimulation of the low-dose, choline-dependent system by morphine and cocaine in acute leukemic cells relative to that observed in normal lymphocytes suggests a possible mechanism of increasing the therapeutic index of nitrogen mustard.

INTRODUCTION

Active transport of the alkylating agent HN2 on the transport carrier for choline has been described in L5178Y murine lymphoblasts (7), rat Walker 256 carcinosarcoma cells (6), and human lymphoid cells (9). HN2 transport in normal and leukemic human lymphoid cells appears to be mediated by 2 components, a low-affinity, high-capacity system that is choline independent at high drug concentrations, and a high-affinity, low-capacity system that is choline dependent at low drug concentrations (10). Activity of the HN2 transport system in L5178Y lymphoblasts appears to be dependent upon proliferative rate of the cells (5) and may also be stimulated by a variety of drugs, including atropine, morphine, and cocaine (4). As an extension of these latter findings, the effect of morphine and cocaine on the activity of the high- and low-dose HN2 transport systems in normal and leukemic human blood cells was studied. Preferential stimulation of HN2 transport by either normal or leukemic cells might have important implications for the clinical use of HN2 as a chemotherapeutic agent.

MATERIALS AND METHODS

Cell Separation. All patients with acute or chronic leukemia were untreated cases. The usual morphological criteria for distinguishing cases of ALL and AML were used, including the appearance of cells after staining with May-Grünwald-Giemsa, peroxidase, periodic acid-Schiff, and Sudan black, as described by Wintrobe (13). Approximately 20 to 30 ml of venous blood were collected from normal subjects or patients with CLL, ALL, or AML and placed in 50-ml sterile centrifuge tubes containing 300 units of heparin. Aliquots of heparinized blood were diluted with 2 volumes of Fischer’s medium (Grand Island Biological Co., Grand Island, N. Y.) containing glutamine, 10% horse serum, penicillin, and streptomycin, and the cells were separated on Ficoll-Hypaque cushions by centrifugation at 400 x g for 40 min in a Sorvall Model GLC-2 centrifuge, by a slight modification of the method of Thorby and Bratlie (12). The separation fluid was made by mixing 10 volumes of 33.9% Hypaque sodium (sodium diatrizoate; Winthrop Laboratories, Aurora, Ontario, Canada) with 24 volumes of 9% aqueous Ficoll (Pharmacia, Uppsala, Sweden), giving a final density of 1.076 to 1.078. Cells appearing at the interface between plasma and separation fluid were carefully removed with Pasteur pipets, washed in sterile 0.85% NaCl solution, and resuspended in Fischer’s medium to give a final cell concentration of 3 to 4 x 10^6 cells/ml.

Cell preparations from normal subjects and patients with
CLL consisted of at least 95% lymphoid cells; those from patients with ALL and AML consisted of 40 to 90% blast cells, the remainder being immature precursor forms and lymphocytes. The final cell suspensions were relatively free of erythrocytes and granulocytes, which sediment to the bottom of the tube. Trypan blue dye exclusion showed 98% of the cells to be viable at the end of the separation.

**Transport Studies.** Transport studies were performed on the above mentioned cell suspensions at 37° by methods described previously (7, 8, 10). Earlier studies of choline and HN2-OH transport by normal and leukemic human cells indicated that uptake was linear for at least 60 min (10); thus, in all experiments, incubation times were routinely set at 60 min.

[1,2-14C]Choline chloride (specific activity, 6.2 mCi/mmole) was obtained from New England Nuclear, Boston, Mass., and HN2-[1,2-14C]2-chloroethyl (specific activity, 3.1 mCi/mmole) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo.; the derivative HN2-OH was prepared by alkaline hydrolysis of the parent compound in 0.1 N NaOH at 60° for 2 hr and was preferred to HN2, in that transport could be studied without the complication of alkylation reactions (7, 8). The effect of morphine and cocaine at a concentration of 50 μM on the transport of HN2-OH and choline at a concentration of 10 μM was evaluated in lymphocytes of normal subjects and patients with CLL and in immature and blast cells of patients with ALL and AML. Studies with HN2-OH acting as transport substrate were a measure of the high-dose (10 to 200 μM), choline-independent transport system, and those using choline as substrate were an index of the low-dose (2 to 10 μM), choline-shared transport component (10).

Drug and transport substrate were added simultaneously to cell cultures; uptake, expressed as cell/medium distribution ratio of substrate, was determined simultaneously in the absence and presence of drug, as described previously (4, 7, 8, 10). Stimulation of uptake of transport substrate was considered significant if the p value was less than 5% by unpaired 2-tailed t test. Incubations were terminated by rapid chilling to 4° and by centrifugation of the cells through a layer of 0.25 M sucrose to remove extracellular radioactivity. The washed cells were solubilized in 0.5 N NaOH, and radioactivity was determined by liquid scintillation spectrometry. Cell size was determined in a Coulter Model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.) 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.). This technique measures mean cell volume and does not take into account variability of blast cell size, which may occur in human acute leukemia. Cell/medium distribution ratios were based on the radioactivity calculated per cell volume relative to that of an equivalent volume of extracellular medium as described previously (4–8, 10).

**RESULTS**

A dose-response curve of HN2-OH transport by normal lymphocytes in the presence of cocaine at a concentration range of 5 to 500 μM was bell shaped, with maximal stimulation occurring at a cocaine concentration of 50 μM, as shown in a typical study (Chart 1). The contour of this dose-response curve was similar to that previously observed for HN2-OH and choline transport by L5178Y lymphoblasts in the presence of cocaine and morphine (4). Accordingly, in all subsequent experiments, the dose of stimulant drug was arbitrarily set at 50 μM, and that of transport substrate, at 10 μM.

**Normal Lymphocytes.** As in previous studies, transport of [1,2-14C]HN2-OH and [1,2-14C]choline chloride by normal lymphocytes proceeded uphill against a concentration gradient. In normal lymphocytes, the cell/medium ratio (mean ± S.E.) for HN2-OH transport in 9 subjects was 14.9 ± 1.0, and that for choline transport in 12 individuals was 15.2 ± 0.9. HN2-OH transport was stimulated by morphine in 8 of 9 individuals and by cocaine in 5 of 9 subjects. Stimulation (mean ± S.E.) of HN2-OH uptake by morphine relative to control uptake was 240.1 ± 14.8%, and stimulation by cocaine was 155.8 ± 8.0%; both effects were highly significant (paired 2-tailed t test, p < 0.005). Conversely, choline transport by normal lymphocytes was minimally stimulated by morphine (111.7 ± 2.3%) and was not affected by cocaine (Table 1).

In normal lymphocytes, morphine stimulation of HN2-OH transport (240.1 ± 14.8%) was much greater than that of choline transport (111.7 ± 2.3%), and the difference was highly significant (p < 0.001). A similar comparison of the effect of cocaine on HN2-OH (155.8 ± 8.0%) and choline transport (101.0 ± 1.4%) by normal lymphocytes was also highly significant (p < 0.001).

**CLL.** Transport of [1,2-14C]HN2-OH and [1,2-14C]choline chloride by lymphocytes from patients with CLL occurred against a concentration gradient. The mean cell/medium ratio for HN2-OH transport in 10 patients with CLL was 5.1 ± 0.5, and that for choline transport in 8 patients was 9.8 ± 1.8. HN2-OH transport was significantly stimulated by mor-
phine in 9 of 10 patients and by cocaine in 7 of 10 subjects. HN2-OH transport in the presence of morphine was 170.0 ± 9.6% of control uptake, and this difference was statistically significant (paired 2-tailed t test, p < 0.01); mean stimulation by cocaine was 128.3 ± 5.2%, and this difference was also significant (paired 2-tailed t test, p < 0.05). However, choline uptake by CLL lymphocytes was not significantly altered by either morphine or cocaine (Table 1).

For HN2-OH transport, both morphine and cocaine had a more profound effect on normal cells than on CLL lymphocytes, and the differences were highly significant (Table 1). For choline transport, stimulation by morphine was observed only in normal lymphocytes and the difference relative to that observed in CLL lymphocytes was statistically significant (p < 0.001); cocaine had a slight inhibitory effect on choline transport in CLL lymphocytes relative to normal cells and this difference was statistically significant (p < 0.01).

The effect of morphine on HN2-OH transport by CLL lymphocytes was greater than that observed on choline transport, and the difference was highly significant (p < 0.001); a similar difference in the effect of cocaine on HN2-OH and choline transport was also highly significant (p < 0.001).

ALL. Transport of [1,2-14C]HN2-OH and [1,2-14C]choline chloride by lymphoblasts of patients with ALL proceeded “uphill” against a concentration gradient. The mean cell/medium ratio for HN2-OH transport in 5 patients with ALL was 20.5 ± 4.4, and that for choline transport in 2 patients was 12.3 ± 0.1. HN2-OH transport was significantly stimulated by morphine in all 5 patients studied and by cocaine in 3 of the 5 subjects. HN2-OH transport in the presence of morphine was 140.0 ± 3.5% higher than control uptake, and this difference was statistically significant (paired 2-tailed t test, p < 0.05). Cocaine stimulated HN2-OH uptake by lymphoblasts 115.5 ± 3.6%, but this effect was not statistically significant. Choline transport by ALL lymphoblasts was significantly stimulated by morphine and cocaine in the 2 subjects studied; transport was increased 139.9 ± 1.6% by morphine and 120.3 ± 2.6% by cocaine, and the effect of both drugs was statistically significant (paired 2-tailed t test, p < 0.05).

Stimulation of HN2-OH transport by morphine and cocaine was more marked in normal lymphocytes than in ALL lymphoblasts and, in each case, the differences were highly significant (p < 0.001), as shown in Table 1. Conversely, stimulation of choline transport by both drugs was greater in ALL lymphoblasts than in normal lymphocytes, and these differences were also highly significant (p < 0.001).

Both HN2-OH and choline transport by ALL lymphoblasts was stimulated to a comparable degree by morphine and cocaine. This situation contrasted sharply with the findings for HN2-OH and choline transport by lymphocytes from normal individuals and patients with CLL, in whom HN2-OH transport was markedly stimulated with little or no effect on choline transport (Table 1).

AML. Uptake of [14C]HN2-OH by peripheral blood cells in 10 patients with AML occurred uphill against a concentration gradient of 9.24 ± 0.9; the cell/medium distribution ratio of choline in cells from 4 patients was 13.8 ± 2.4. HN2-OH transport by cells from patients with AML was significantly stimulated by morphine in 7 of 10 subjects and by

<table>
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<tr>
<th>Subjects</th>
<th>Morphine (%)</th>
<th>p</th>
<th>Cocaine (%)</th>
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<tr>
<td>HN2-OH uptake</td>
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<td>Normal (9)</td>
<td>240.1 ± 14.8</td>
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<td>155.8 ± 8.0</td>
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<tr>
<td>CLL (10)</td>
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<td>128.3 ± 5.2</td>
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<td>ALL (5)</td>
<td>140.0 ± 3.5</td>
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<td>115.5 ± 3.6</td>
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<tr>
<td>AML (10)</td>
<td>143.7 ± 7.4</td>
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<td>105.0 ± 3.4</td>
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<tr>
<td>Choline uptake</td>
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<tr>
<td>Normal (12)</td>
<td>111.7 ± 2.3</td>
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<td>101.0 ± 1.4</td>
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<td>CLL (8)</td>
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<td>ALL (2)</td>
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<td>120.3 ± 2.6</td>
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<tr>
<td>AML (4)</td>
<td>134.4 ± 7.5</td>
<td>&lt;0.001</td>
<td>113.7 ± 3.0</td>
<td>&lt;0.001</td>
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</tbody>
</table>

* For each group of subjects, substrate uptake (mean ± S.E.) in the presence of morphine or cocaine has been compared to control uptake by a paired, 2-tailed t test, and the results of this analysis are presented in the text.

* The p value represents a comparison of stimulation of substrate uptake by morphine or cocaine in each group of patients relative to the stimulation observed in normal subjects, using an unpaired, 2-tailed t test.

* Numbers in parentheses, number of subjects studied. For each individual experiment, determinations were performed in quadruplicate. The number of individuals in each group in which stimulation of uptake was significant by unpaired, 2-tailed t test is presented in the text.

* The effect of cocaine on HN2-OH uptake by cells from patients with AML was evaluated in 9 subjects.
cocaine in only 1 of 9 subjects. HN2-OH transport in the presence of morphine was 143.7 ± 7.4% of control uptake, and this difference was statistically significant (paired 2-tailed t test, p < 0.01). However, HN2-OH uptake in the presence of cocaine was only 105.5 ± 3.4% of control uptake, and this difference was not significant. Morphine significantly stimulated choline uptake in 3 of 4 patients with AML by 134.4 ± 7.5%, but the overall effect in the group fell short of statistical significance; choline transport was not significantly altered by cocaine in any of the 4 patients studied.

A comparison of the effect of morphine and cocaine on HN2-OH and choline transport by cells from patients with AML resembled the observations noted in patients with ALL (Table 1). Stimulation of HN2-OH transport by morphine and cocaine was greater in normal lymphocytes than in cells from patients with AML, and for each drug the differences were highly significant (p < 0.001); conversely, the effect of morphine and cocaine on choline transport was more marked in cells from patients with AML, and these differences were also highly significant (p < 0.001).

With the exception of choline transport by CLL lymphocytes, HN2-OH and choline transport by cells from subjects in each of the 4 groups was more responsive to morphine than to cocaine, and the differences were all highly significant (p < 0.001).

DISCUSSION

Transport of HN2-OH and choline by L5178Y lymphoblasts in vitro was previously shown to be stimulated by morphine, cocaine, and atropine (4). The effect appeared to be on unidirectional influx of substrate, since the stimulating drugs had no effect on efflux of transport substrate. Kinetic studies showed the dominant effect was an increase in $V_{\text{max}}$, suggesting that the mechanism of stimulation may involve exposure of new carrier sites and/or allosteric changes in the configuration of existing carriers leading to increased carrier mobility. More pronounced stimulation of choline transport relative to that of HN2-OH was noted; the explanation suggested for this finding was that choline is the native and preferred transport substrate (4, 7).

The potential relevance of these experimental findings lies in the knowledge that patients with malignant disease often receive many drugs for supportive therapy simultaneously with antitumor chemotherapy and that increasing numbers of patients with malignant disease are being treated with combination chemotherapy regimens. Such multiple drug programs create a situation that might lead to unexpected synergistic or antagonistic pharmacological combinations, part of which might be explained by effects on drug transport. Others have shown that vincristine stimulates methotrexate uptake by L1210 cells (14) and by human AML cells in vitro (1). Stimulation of methotrexate transport in vitro was associated with enhanced survival of mice bearing L1210 leukemia when treated with vincristine and methotrexate (14). Conversely, hydrocortisone inhibited not only the uptake of methotrexate by L1210 cells but also its biological effect, as measured by the deoxuryridine suppression test, in L1210 and human bone marrow cells (2).

In the present study, morphine and cocaine markedly stimulated HN2-OH uptake by lymphocytes in normal individuals and patients with CLL, with little or no effect on choline transport. These observations lend further support to the concept that HN2-OH transport by human lymphocytes occurs by 2 components: 1, operative at high HN2 concentrations, is a low-affinity, high-capacity system that is choline independent; and the 2nd, active at low substrate concentrations, is a high-affinity, low-capacity system that is shared with choline (10). Thus it would appear that, in lymphocytes from normal individuals and patients with CLL, the high-dose, choline-independent HN2-OH transport system is stimulated by morphine and cocaine, with little or no effect on the low-dose, choline-shared pathway. It is doubtful whether these observations are clinically significant, since the high-dose system involves drug transport at a concentration range of 10 to 200 μM (10). It is unlikely that such drug concentrations would be attained with the usual pharmacological doses of HN2, since HN2 is a highly reactive compound and the active form is removed from the blood in a few minutes (3, 9, 11).

In patients with ALL and AML, both HN2-OH and choline transport were stimulated by morphine and to a lesser extent by cocaine (Table 1). In this respect, cells from patients with acute leukemia responded more like L5178Y lymphoblasts (4) than did lymphocytes from normal individuals or patients with CLL. For HN2-OH transport, stimulation by morphine and cocaine was more pronounced in normal lymphocytes than in leukemic blast cells and although, in theory, this might lead to a reduction in therapeutic index, this is considered improbable since the concentrations of HN2 probably are not pharmacological, as discussed above. Conversely, stimulation of choline transport by morphine and cocaine was more marked in leukemic blast cells than in normal lymphocytes. Unlike the findings with high-dose transport, these results are of potential clinical importance. First, the drug concentrations attainable clinically are more likely to fall within the low concentration range (i.e., less than 10 μM). Second, preferential stimulation of the low-dose, choline-dependent system, particularly by morphine in acute leukemic cells, suggests a possible mechanism of increasing the therapeutic index of HN2. Finally, these findings suggest that further studies are warranted to determine whether morphine or cocaine might preferentially increase the cytoxic effect of HN2 on neoplastic cells in vivo; initially, this might be achieved by evaluating such drug combinations in mice with L5178Y leukemia or in rats with Walker 256 carcinosarcoma.

ACKNOWLEDGMENTS

The author thanks Marjorie Lee for technical assistance and Dorothy Faulkner for typing the manuscript.

REFERENCES

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