Cytosine Arabinoside Influx and Nucleoside Transport Sites in Acute Leukemia

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ABSTRACT Although cytosine arabinoside (araC) can induce a remission in a majority of patients presenting with acute myeloblastic leukemia (AML), a minority fail to respond and moreover the drug has less effect in acute lymphoblastic leukemia (ALL). The carrier-mediated influx of araC into purified blasts from patients with AML, ALL, and acute undifferentiated leukemia (AUL) has been compared to that of normal lymphocytes and polymorphs. Blasts showed a larger mediated influx of araC than mature cells, since mean influxes for myeloblasts and lymphoblasts were 6- and 2.3-fold greater than polymorphs and lymphocytes, respectively. Also, the mean influx for myeloblasts was fourfold greater than the mean for lymphoblasts. The number of nucleoside transport sites was estimated for each cell type by measuring the equilibrium binding of [³H]nitrobenzylthioinosine (NBMPR), which inhibits nucleoside fluxes by binding with high affinity to specific sites on the transport mechanism. The mean binding site numbers for myeloblasts and lymphoblasts were 5- and 2.8-fold greater, respectively, than for the mature cells of the same maturation series. The mean number of NBMPR binding sites for myeloblasts was fourfold greater than for lymphoblasts. Patients with AUL were heterogeneous since blasts from some gave values within the myeloblastic range and others within the lymphoblastic range. The araC influx correlated closely with the number of NBMPR binding sites measured in the same cells on the same day. Transport parameters were measured on blasts from 15 patients with AML or AUL who were then treated with standard induction therapy containing araC. Eight patients entered complete remission, while seven failed therapy, among whom

were the three patients with the lowest araC influx (<0.4 pmol/10⁷ cells per min) and NBMPR binding (<3,000 sites/cell) for the treated group. In summary, myeloblasts have both higher araC transport rates and more nucleoside transport sites than lymphoblasts and this factor may contribute to the greater sensitivity of AML to this drug. AraC transport varied >10-fold between leukemic blasts and normal leukocytes, but transport capacity related directly to the number of nucleoside transport sites on the cell. Finally, low araC transport rates or few NBMPR binding sites on blasts were observed in a subset of patients with acute leukemia who failed to achieve remission with drug combinations containing araC.

INTRODUCTION

First remissions can be induced in approximately half the patients with acute myeloblastic leukemia (AML)¹ by therapy with drug combinations containing cytosine arabinoside (araC). It is generally considered that the conversion of araC to araCTP is central for the cytotoxic effect of this drug since araCTP is known to inhibit the synthesis of DNA (1-5). Resistance of cells to araC has been considered to arise from deficiency in araC phosphorylation by deoxycytidine kinase possibly coupled with increased degradation of araC by cytidine deaminase (6-8). However, these enzymatic activities have not proven to be useful predictors of clinical response to araC therapy (9, 10). Permeation of araC is an essential prerequisite for its metabolic conversions but there is no information on this process in fresh leukemic cells. Movement of nu-

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¹ *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; AMML, acute myelomonocytic leukemia; araC, 1-β-D-arabinofuranosylcytosine; araCTP, the 5'-triphosphate of araC; AUL, acute undifferentiated leukemia; NBMPR, nitrobenzylthioinosine or 6-[(4-nitrobenzyl]thio)-9-β-D-ribofuranosyl purine.

cleoside and many of their analogs across the plasma membrane in many cell types is mediated by a nucleoside specific transport mechanism. Cytosine arabinoside is a substrate for this carrier system (11–15) that is reversible, nonconcentrative, and has a broad specificity for pyrimidine and purine nucleosides. However, the affinity of the nucleoside carrier in leukemic cells is less for araC than for its naturally occurring pyrimidine analog, deoxycytidine.²

A number of potent inhibitors of nucleoside transport have been identified such as dipyridamole and nitrobenzylthioinosine (NBMPR). These compounds bind with high affinity to specific sites on the exterior of the cell, such that occupancy of these sites by inhibitor blocks nucleoside transport. Several studies suggest that the number of NBMPR binding sites on cell membranes can be equated with the number of functional nucleoside transport sites. Strong evidence comes from work on the genetic polymorphism of nucleoside-permeable or impermeable sheep erythrocytes. Nucleoside-permeable erythrocytes possess high affinity NBMPR binding sites but such sites were absent from erythrocytes lacking capacity for nucleoside transport (16). Moreover in human erythrocytes a direct proportionality has been shown between the fractional inhibition of uridine transport and the occupancy of the total available NBMPR binding sites (17).

The present study has examined whether variability in the nucleoside permeability of leukemic blasts may offer an explanation for the resistance of some leukemias to araC. Both the influx of araC as well as the number of NBMPR binding sites have been measured on blasts prepared from fresh peripheral blood. In a group of 15 patients these transport parameters have been correlated with the clinical response to combination chemotherapy containing araC.

METHODS

Materials. Imidazole-buffered saline (145 mM NaCl, 5 mM KCl, 5.0 mM imidazole-Cl, 1.0 mM MgCl₂, 5.0 mM glucose, pH 7.4) was ultrafiltered through Millipore Millex 0.22- μ m filter units (Millipore Corp., Bedford, Mass.) before use. All washing and incubation media contained deoxyribonuclease (10 μ g/ml, Sigma Chemical Co., St. Louis, Mo.) to reduce the tendency of leukocytes to clump. Carbonyl iron (grade SF) of nominal particle Diam 3 μ m was obtained from GAF Corp., New York. Dextran T500 and Ficoll-Isopaque (*d*, 1.077 g/ml) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Di-*n*-butyl-phthalate (British Drug Houses, Poole, England) and di-*n*-octyl phthalate (Ajax Chemicals Ltd., Sidney, Australia) of *d* 1.044 and 0.986 g/ml, respectively, were blended 4:1 (vol/vol) yielding an oil mixture of *d* 1.032 g/ml. Dow Corning DC702 and DC200 oils (Dow Corning Corp., Midland, Mich.) of *d* = 1.07 and 0.873 g/ml and viscosities 45 and 2 cs, respectively, were

blended 4:1 (vol/vol) yielding an oil mixture of *d* = 1.031 g/ml ("silicone oil mix"). NBMPR synthesized as described by Paul et al. (18) was stirred vigorously with imidazole-buffered saline for 4 h at 18–20°C to yield a saturated solution of ~ 20 μ M. This solution was prepared on the day of each experiment. [G-³H] NBMPR was from Morevek Biochemicals Ltd., Brea, Calif. and high-pressure liquid chromatography analysis showed $\sim 75\%$ purity of this labeled inhibitor. [5-³H]araC and [U-¹⁴C]sucrose in sterile 3% aqueous ethanol were from the Radiochemical Centre, Amersham, England. Solutions of 5.0 mM cytosine arabinoside (Upjohn Co., Kalamazoo, Mich.) in isotonic saline were mixed with 0.1 vol of the appropriate tritiated stock, the exact concentrations of the final solutions (4.1–4.5 mM) being confirmed spectrophotometrically at pH 1 using a molar extinction coefficient of 1.32×10^4 at 280 nm. Scintillant fluid consisted of 2 vol toluene, 1 vol Triton X-100 plus 4 g PPO (Packard Instruments Co., Downers Grove, Ill.) per liter, used in a ratio of 1 ml aqueous phase to 10 ml scintillant (19).

Patient data. Patients with acute leukemia had peripheral leukocyte counts of 13,500–300,000/ μ l, (60–99% blasts) and were diagnosed from morphology and cytochemistry of blood and bone marrow specimens. All samples were collected with informed consent before the initiation of any chemotherapy. Patients ranged in age from 18 mo to 70 yr. Acute lymphoblastic leukemia (ALL) was only diagnosed when the periodic acid-Schiff stain was positive for blasts of lymphoid morphology. Two of six patients with ALL had blasts that were immunologically typed as T cells. AML was diagnosed when 3% or more of blasts gave a positive peroxidase reaction or Auer rods were seen (20). AMML or monocytic leukemia were diagnosed when the proportion of promonocytes and monocytes in blood or marrow exceeded 20% and these diagnoses were confirmed by a positive nonspecific esterase reaction or elevation of serum lysozyme >10 μ g/ml. Because AML and acute myelomonocytic leukemia (AMML) are derived from a common stem cell, these two leukemias were analyzed in the one category. Acute undifferentiated leukemia (AUL) was diagnosed when no blast differentiation was apparent and the peroxidase, periodic acid Schiff, and nonspecific esterase reactions were all negative.

Treatment protocol and definition of response. Patients with AML (*n* = 5), AMML (*n* = 4), acute monocytic leukemia (*n* = 1), or AUL (*n* = 5) were treated with standard induction therapy consisting of continuous intravenous araC (100 mg/m² per d) for 7 d plus daunorubicin (45 mg/m²) on days 1, 2, and 3 (21). Duration of araC infusion was shortened to 5 d for second and subsequent courses (22). All patients received two or more courses except patient M.F. who declined therapy after 4 d of araC because of intense nausea. Criteria for response were those of the acute leukemia group B (23). Patients in complete remission were asymptomatic, and had nondiagnostic bone marrow morphology and normal peripheral blood counts. Patients with partial remission were also asymptomatic and had a normal peripheral blood count.

Lymphocyte and blast isolation. Venous blood from healthy subjects (120–180 ml) and leukemic patients (10–30 ml) was defibrinated by shaking with acid-washed glass beads (2 mm Diam) in a stoppered flask. When platelet counts were $<10,000/\mu$ l blood was drawn into heparin anticoagulant. Normal blood was diluted with 2 vol saline and incubated with carbonyl-iron (0.5 mg/ml) with slow agitation at 37°C for 40 min to remove phagocytic mononuclear cells (24). Blasts and lymphocytes were separated by isopycnic sedimentation on a Ficoll-Isopaque gradient at 400 g at 18–20°C for 30 min in polycarbonate tubes. Cells at the

² Sawyer, W. H., S. P. Jones, and J. S. Wiley. Manuscript in preparation.

interface were harvested and washed thrice in buffered saline.

In some normal lymphocyte preparations erythrocyte contamination was as much as 35% of the total cell count, as quantitated by cell counts with and without saponin (Zaponin; Coulter Electronics, Ltd., Dunstable, England) an erythrocyte lysing agent. Erythrocytes were depleted by cold hypotonic shock lysis of the suspension (25). To 2 ml cell suspension in isotonic buffered saline were added 6 ml of cold distilled water (yielding 0.25 times isotonicity) followed after 30 s by the rapid addition of 2 ml of 600 mM NaCl to restore isotonicity. Leukocytes were separated from hemolyzed material by centrifugation at 150 g for 5 min followed by aspiration of erythrocyte ghosts on the surface of the leukocyte layer. After two such treatments the final erythrocyte contamination was always <5–10% of the total cell count.

Polymorphonuclear leukocyte preparation. Venous blood (60–180 ml) from normal donors was defibrinated, mixed with 0.1 vol 4% dextran T500 in 150 mM NaCl, and left to stand at 18–20°C for 60–75 min. The supernate contained mainly leukocytes, and was centrifuged over a Ficoll-Isoopaque density gradient as above. The cell pellet of polymorphs was resuspended in 2 ml of imidazole-buffered saline and residual erythrocytes lysed by the addition of 18 ml ice-cold water for 30 s, followed by restoration of isotonicity with 20 ml 300 mM NaCl. Polymorphs were washed thrice in buffered saline.

Cell suspensions. Cells were suspended at $0.5\text{--}2.5 \times 10^7$ cells/ml in imidazole-buffered saline. Cell counts were performed on a Coulter model DN electronic particle counter with a 100- μ m orifice. Homogeneity and cell morphology were checked on a cytocentrifuge preparation for each experiment. Normal lymphocyte preparations contained $95 \pm 2\%$ (SD) lymphocytes, $3 \pm 1\%$ monocytes and $2 \pm 1\%$ polymorphs (24), while acute leukemic preparations always contained >95% blasts.

Measurement of [3 H]araC influx. The cell suspension (1.2 ml) was incubated in stoppered plastic tubes at 20.0°C for 5 min, [3 H]araC (5–180 μ M) added at zero time and the suspension gently agitated. In some experiments samples (1.0 ml) were removed at 30 s and added to microfuge tubes containing 0.1 ml 20 μ M NBMPR, to stop any further cell uptake of labeled nucleoside (“inhibitor-stop” technique). Phthalate oil mix (0.3 ml) was added and the cells centrifuged under the oil layer in an Eppendorf microcentrifuge at 8,000 g for 4 min. In other experiments samples were removed and the influx was terminated at 30 s by simply centrifuging the cells through the phthalate-oil mixture (“oil-stop” technique). The supernatant above the oil interface was sampled and counted to determine the concentration of araC. The remainder of the supernatant was aspirated, the walls of the microfuge tubes washed thrice with H₂O, and most of the oil removed with the final washing. Cell pellets were solubilized in 0.5 ml 0.5 N NaOH, scintillation fluid added and the vial contents were acidified and counted. The specific activity of [3 H]araC was also measured to allow the calculation of [3 H]araC uptake in picomoles per 10^7 cells. The nonfacilitated uptake of [3 H]araC was always measured in parallel incubations of cell suspensions containing 3 μ M NBMPR. This concentration of NBMPR is known to abolish the saturable (facilitated) component of nucleoside entry in HeLa as well as other cells (26, 27). Facilitated influx was taken as the difference between the uptake of araC in the absence and the presence of 3 μ M NBMPR. All uptake values were corrected for araC in the extracellular space of the cell pellet (0.15–0.30 μ l/ 10^7 cells), measured in

each experiment as the [14 C]sucrose space. This correction accounted for 10–50% of the total [3 H]araC present in the cell pellets.

Measurement of [3 H]NBMPR binding. Cell suspensions were preincubated at 37°C for 5 min, [3 H]NBMPR (0.1–6.0 nM) added, and tubes gently mixed. Aliquots of 1.0 ml were taken after 5 min incubation at 37°C, layered over 0.3 ml silicone-oil mix, and cells separated by centrifuging at 8,000 g for 4 min. Cell pellets were counted as above. The non-specific binding of [3 H]NBMPR was measured in parallel incubations of cells with unlabeled NBMPR (3 μ M) added before the [3 H]NBMPR addition. Specific binding was taken as the difference between the isotope binding to cells incubated with [3 H]NBMPR and those incubated with unlabeled NBMPR plus [3 H]NBMPR. The correction for [3 H]NBMPR trapped in the extracellular space of the cell pellet was always <5% of the total pellet counts.

Kinetic and Scatchard analyses. The kinetics of facilitated influx of araC into leukocytes and blasts was well-described by a Michaelis-Menten treatment. The kinetic data were analyzed by means of plots of S/V vs. S and of V vs. V/S to which linear regression lines were fitted. Concordance was observed between estimates of the kinetic parameters from the two methods that were used to calculate araC influx at 1 μ M extracellular concentration (V 1 μ M).

The specific binding of [3 H]NBMPR was analyzed by Scatchard plots as described by Edsall and Wyman (28) and the maximum number of binding sites per cell was obtained by extrapolation of the regression line to the abscissa, while the dissociation constant (K_d) was the reciprocal of the association constant given by the slope of the regression line.

Intracellular water space. Cell suspensions were incubated for 5 min at 37°C with $^3\text{H}_2\text{O}$ (5 μ Ci/ml) and aliquots layered over phthalate oil mix, and centrifuged at 8,000 g for 4 min. The trapped extracellular space was determined with [3 H]sucrose in parallel tubes. Cell pellets were counted and intracellular water space calculated (29).

Statistics. Mean values ± 1 SD are shown unless otherwise stated and the significance of differences between means were analyzed by a Student's *t* test. Regression lines were fitted by the method of least squares.

RESULTS

AraC uptake over short time intervals. Analysis of nucleoside influx is only valid if the initial rates of araC uptake into cells is determined. The uptake of araC into blasts incubated with 1 μ M araC for 10–60 s at 20°C was measured using an “inhibitor-stop” technique. Total uptake of nucleoside was linear with incubation time to 60 s and the nonfacilitated component, defined by the uptake in the presence of 3 μ M NBMPR was <22% of the total uptake (Fig. 1). With 1 μ M nucleoside present extracellularly, the relative proportion of nonfacilitated to total araC uptake averaged $15 \pm 4\%$ for AML, $21 \pm 7\%$ for ALL, $11 \pm 3\%$ for AUL, $16 \pm 2\%$ for normal lymphocytes and $22 \pm 9\%$ for normal polymorphs (mean $\pm$ SEM, $n = 4\text{--}11$). None of these values differed significantly so that in all cell types studied the major and constant fraction of araC entry is by a facilitated diffusion mechanism. The influx of araC in all subsequent experiments was taken

FIGURE 1 Time-course of nucleoside uptake by leukemic myeloblasts. [^3H]araC was added at final concentration of $1.0\ \mu\text{M}$ to the cell suspension that had been preincubated at 20°C for 5 min in the absence or presence of $3\ \mu\text{M}$ NBMPR.

as the NBMPR-sensitive component of [^3H]araC uptake during 30 s of incubation at 20°C .

Influx of [^3H]araC into leukocytes. The constant intravenous infusion of araC at therapeutic doses ($70\text{--}130\ \text{mg}/\text{m}^2$ per d) achieves plasma levels of $0.3\text{--}1.1\ \mu\text{M}$ (30). For this reason the influx of araC into blasts and mature blood cells was compared at extracellular concentration of $1\ \mu\text{M}$ nucleoside. Although immature blasts of all types showed great variability in araC influx (Fig. 2) the mean rate for blasts of myeloblastic and myelomonocytic leukemias ($2.04\ \text{pmol}/10^7$ cells per min) was significantly greater than the means for undifferentiated ($1.13\ \text{pmol}/10^7$ cells per min) or lymphoblastic leukemias ($0.52\ \text{pmol}/10^7$ cells per min; $P < 0.05$ and $P < 0.001$, respectively). An even more striking difference was observed between mature leukocytes and blasts of the same maturation series. Although lymphocytes and polymorphs had similar araC influxes (0.23 and $0.33\ \text{pmol}/10^7$ cells per min, respectively) these values were only 45 and 16% of araC influxes for lymphoblasts and myeloblasts, respectively.

Binding of [^3H]NBMPR to leukocytes. Cells were incubated with graded concentrations (0.1 to $8.5\ \text{nM}$) of [^3H]NBMPR at 37°C in the presence and absence of $3\ \mu\text{M}$ unlabeled NBMPR and the difference in binding under these two conditions was taken as the specific component of [^3H]NBMPR binding. This specific component was time dependent, reached a maximum within 5 min incubation, and then remained constant up to 20 min at 37°C . The specific binding of [^3H]NBMPR at 5 min incubation was taken as the equilibrium level, which showed a saturable dependence on NBMPR concentration (Fig. 3). Analysis

yielded linear Scatchard plots suggesting that the inhibitor interacts with a single class of binding site (Fig. 4). The affinity of NBMPR binding was calculated from the slope of the Scatchard plots and was similar in all cell types studied (K_d 0.8 to $1.6\ \text{nM}$). Values for the maximal number of binding sites for various cell types are shown in Fig. 5. Blasts showed a 12-fold variation in binding site numbers and the mean for AML and AMML cells ($12,220 \pm 5,210$ sites/cell) was significantly greater than the means for AUL cells ($6,030 \pm 3,040$ sites/cell) or ALL cells ($3,100 \pm 940$ sites/cell; $P < 0.02$). Consistent values were obtained for NBMPR binding sites in the cells from one patient on different days. A patient (P.B.) with AUL gave repeated values of $3,030$, $2,380$, and $2,200$ sites/cell over a 6-mo interval during which the leukocyte count ranged from $13,500$ to $86,000/\mu\text{L}$. The latter two values for binding sites were obtained from blood samples containing significant numbers of lymphocytes ($10\text{--}30\%$) that may have accounted for the lower estimates. Another patient with AUL gave repeated values of

FIGURE 2 Influx of araC into blasts from patients with various leukemias and normal lymphocytes and polymorphs. Influx at $1\ \mu\text{M}$ concentration was derived from the kinetic parameters of facilitated araC influx. The two patients with T cell ALL are shown by open circles, while the other ALL patients had null cell typing. Mean values ± 1 SD are shown on the histogram.

FIGURE 3 Dependence of NBMPR binding at equilibrium on the free concentration of ligand. Blasts were incubated for 5 min at 37°C with [3 H]NBMPR at the shown concentrations after a preincubation for 5 min in the absence, ●, or presence, ■, of 3 μ M unlabeled NBMPR. Specific binding, ○; represents the difference between binding in absence and presence of excess unlabeled NBMPR.

6,730 and 5,930 sites/cell over several months. Normal lymphocytes and polymorphs gave mean binding site numbers of $1,120 \pm 610$ and $2,270 \pm 690$ sites/cell, respectively, which were 36 and 19% of the values obtained for the blasts of the same maturation series. A mixed population of promyelocytes-myelocytes-metamyelocytes was separated from the peripheral blood of a patient with the accelerated phase of chronic myeloid leukemia. A mean value of NBMPR binding of 7,180 sites/cell was obtained that was intermediate between the value for myeloblasts and polymorphs.

Correlation of araC influx with specific NBMPR binding sites. Concurrent measurement of [3 H]araC influx and [3 H]NBMPR binding were made on each preparation of lymphocytes, polymorphs or blasts. Fig. 6 shows that a close correlation exists between the influx of araC at an extracellular concentration of 1 μ M nucleoside and the maximum number of NBMPR binding sites per cell ($r = 0.87$; $P < 0.01$). Thus much of the great variability in araC influx may relate to the number of NBMPR binding sites present in the different cells.

Cell volume and specific NBMPR binding sites. In each experiment the intracellular water space of blasts or leukocytes was measured as an index of cell size (29). The intracellular water space of blasts, $1.95 \pm 0.3 \mu\text{l}/10^7$ cells ($n = 10$) was not different from mature polymorphs ($1.72 \mu\text{l}/10^7$ cells) but was greater than lymphocytes ($1.44 \pm 0.24 \mu\text{l}/10^7$ cells, $n = 5$). There was a weak correlation between the number of NBMPR

FIGURE 4 Scatchard analysis of specific NBMPR binding to blasts prepared from three patients with different acute leukemias. Regression lines have been fitted by the method of least squares. The intersect on the x-axis estimates the maximum numbers of binding sites.

binding sites on blasts and either intracellular water space ($r = 0.68$; $P < 0.02$) or the minimum surface area of the cell³ calculated from this intracellular water space ($r = 0.67$; $P < 0.05$). Thus the size of a cell or its surface area may be factors that contribute to the large variability in NBMPR binding site numbers between different blast types.

Relation of clinical response to araC influx. Transport parameters for araC were studied in 15 patients at presentation who were then commenced on standard induction therapy with 7 d of continuous araC plus three daily doses of daunorubicin (21, 22). Eight patients achieved complete remission while another five failed, despite completing two or more courses of the combination containing araC. Two patients reached partial remission, one after two courses and another (M.F.) after only 4 d of araC although she then declined further treatment because of nausea. The three patients with araC influx into their peripheral blood blasts of $< 0.4 \text{ pmol}/10^7$ cells per min all failed induction therapy (Fig. 7). The number of NBMPR binding sites on blasts from two of these three patients (P.B. and E.L.) was 2,540 and 2,040 sites/cell, respectively, which were the lowest site densities among the treated patients. Binding of NBMPR to blasts from the third patient was not measured. The other two patients who

³ Minimum surface area of the cell was calculated from the intracellular water space assuming 75% water content and spherical geometry.

FIGURE 5 Maximum number of NBMPR binding sites on blasts from patients with various leukemias and normal lymphocytes and polymorphs. Two patients with T cell ALL are shown by the open circles, while the other patients with ALL had null typing. Mean values $\pm$ 1 SD are shown on the histogram.

failed induction therapy had a high araC influx into their blasts (1.2 and 1.4 pmol/ 10^7 cell per min). Failure in one was due to death from recurrent septicemia,

FIGURE 6 Correlation between influx of araC at 1 μ M concentration and maximum number of NBMPR binding sites for AML and AMML blasts, ■; AUL blasts, ▲; ALL blasts, ●; normal lymphocytes, ○; normal polymorphs, □. Regression line is fitted by the method of least squares ($r = 0.87$; $P < 0.01$).

FIGURE 7 AraC influx into blasts and response of patients with acute leukemia to induction therapy with an araC plus daunorubicin combination. Five patients with acute myeloid leukemia (T.L., D.W., P.H., T.B., and J.C.), four with acute myelomonocytic leukemia (M.S., H.M., V.P., and T.M.), one with acute monocytic leukemia (J.E.), and five with acute undifferentiated leukemia (M.P., M.F., E.L., P.B. and D.W.) were studied. All patients except M.F. received at least two courses of combination chemotherapy. Response was assessed as complete remission, ○; partial remission, ●; or failure, ■.

and the other died from oliguric renal failure possibly from nephrotoxic effects of antibiotics given for an infection.

DISCUSSION

Large differences in the transport⁴ of araC are apparent between various types of blood cells. The main finding is that the leukemic blasts have a higher influx rate for araC compared with normal mature leukocytes. Fig. 2 shows that the mean influx of araC measured at 1 μ M external concentration was two to six times greater for either lymphoblasts or myeloblasts than for mature cells of the same differentiation series. However in each group studied there was a wide scat-

⁴ Transport refers to the mediated passage of nucleoside molecules across the plasma membrane. Influx is defined as the initial rate of entry of solute i.e., slope of NBMPR-sensitive uptake of nucleoside shown in Fig. 1.

ter of individual values, especially for the AML patients who showed a 10-fold range of influxes. The mean influx for the AUL patients was intermediate between mean values for AML and ALL as might be expected for a heterogeneous group of patients, some of whom had blasts of lymphoid character while others had blasts with myeloblastic features (31). The wide scatter of influx values between individuals was observed both for total araC uptake rates as well as for the NBMPR-sensitive component of araC uptake (influx) that occurs via a facilitated transport mechanism and is the major route by which nucleoside enters the cell. Despite the variability in influx between different cell preparations, the NBMPR-sensitive component of araC uptake always accounted for a relatively constant (78–89%) proportion of the total araC entry rate with 1 μ M external araC concentration. Thus leukemic blasts resemble HeLa and other cultured cells in which the major route of nucleoside entry is by a facilitated diffusion system (26, 27). Why is nucleoside transport greater in immature blasts than in the end product cells of differentiation? The proliferative rate of blasts is clearly greater than their mature descendents and it is known that several membrane transport processes are greater for cells in active division compared with their quiescent counterparts. Both active K⁺ influx and the number of cation pumps increase fourfold after stimulation of 3T3 cells into proliferation with serum, while glucose and phosphate transport rates are also

elevated under the same conditions (32, 33). The high araC influx of proliferating blasts could result either from a greater number of nucleoside carriers or a higher turnover rate of each carrier and these two possibilities were studied.

Equilibrium binding studies of NBMPR to intact cells allows the nucleoside-transport elements in the cell membrane to be directly enumerated (34). Lymphoblasts possessed 2.8-fold more NBMPR binding sites than lymphocytes, while myeloblasts had fivefold more binding sites than polymorphs. A mixed population of promyelocytes, myelocytes, and metamyelocytes had a mean value for NBMPR binding that was intermediate between myeloblasts and polymorphs. Thus, a high number of NBMPR binding sites is a general characteristic of immature blasts and their immediate descendents compared with the more mature counterparts in the sequence of myeloid and lymphoid differentiation. However, myeloblasts had fourfold more binding sites than lymphoblasts and this finding cannot be attributed to differences in maturity or state of cell differentiation. Also, AUL showed a mean binding site density intermediate between the values for myeloblasts and lymphoblasts. Is the higher proliferative rate of blasts a likely explanation for their greater density of NBMPR binding sites? Many types of cultured neoplastic cells of both human and mouse origin have been studied with respect to the density of NBMPR binding sites (Table I). All of the cultured

TABLE I
Direct Enumeration of Transport Inhibitory Sites in Various Cell Types

	Intracellular water	Cell volume	Maximum NBMPR binding	Binding site density	Affinity of binding at equilibrium K_d	Reference
	$\mu\text{l}/10^7 \text{ cells}$	fl	sites/cell	sites/fl. vol	nM	
Lymphocyte	1.44	—	1,124	6.6	1.2	This study
Polymorph	1.72	—	2,271	10.0	1.5	This study
Myeloblast (AML, AMML)	2.56	—	12,220	40.0	1.6	This study
Lymphoblast (ALL)	1.73	—	3,117	15.0	0.8	This study
L5178Y	6.5	—	200,000	249	—	Paterson (unpublished data)
L1210	—	290–410	180,000	514	1.1	Cass (unpublished data)
RPMI 6410	9.6	—	330,000	287	0.5	Paterson et al. (54)
HeLa	13.6	—	150,000	89.3	0.5	Lauzon and Paterson (55)
S49	4.5	—	108,000	194	0.2	Kolassa et al.
						Wiley et al. ⁵ (unpublished data)
RC2a	4.6	—	69,800	123	1.8	

NBMPR binding data for the blood cells above represent means of between 4 and 13 preparations. L5178Y and L1210 are mouse leukemic cell lines; RPMI 6410 is a human B-cell lymphoblastoid cell line; S49 is a mouse T cell lymphoma and HeLa is a human cervical carcinoma cell line. RC2a is a monoblast line derived from a patient with AMML. When Coulter volumes were not available, this parameter was calculated from the intracellular water space assuming cell water of 75–78% by weight and cell density 1.07–1.085.

cells cited in Table I were kept in an exponential phase of growth with doubling times between 12 and 24 h and it is noteworthy that a very high density of NBMPR binding sites between 90 and 500 sites/fl vol appears to be characteristic of cell populations permanently committed to proliferation. In contrast the density of NBMPR binding sites on leukemic blasts was only 15 to 40 sites/fl cell vol (Table I). The growth fraction of leukemic cells is generally considered <1.0 and recent estimates based on nuclear DNA-polymerase activity suggest that less than half the leukemic blasts of marrow are in active proliferation (35, 36). Moreover the growth fraction of blasts from peripheral blood may be still lower, since tritiated thymidine labeling indices are considerably less for circulating than for intramedullary blasts (37, 38). Thus, those blasts that are in the proliferating cycle should have an NBMPR site density well in excess of 30–80 sites/fl, a value that approaches that of cultured cells in exponential growth and is far above the values observed for mature, nondividing lymphocytes and polymorphs (6–10 sites/fl cell vol). While the comparisons in Table I indicate that the proliferative state of the cell population is a major factor in determining the density of NBMPR binding sites, an additional factor of cell volume or surface area should be considered. In 10 measurements on different leukemic blasts, a positive correlation was found between the number of NBMPR binding sites per cell and either the intracellular water space or cell surface area calculated from the former on the assumption of 75% cell water and spherical cell geometry. These correlations do suggest that cell size or cell surface area may also be a determinant of the number of NBMPR sites per cell. Whether differences in cell surface area may account for the greater density of NBMPR binding sites on myeloblasts than on lymphoblasts is unknown.

It is established that araC is a substrate for the nucleoside-specific transport mechanism in the cell membrane (12–14, 39) so that transport rate of araC should relate to the density of transport elements in the cell membrane. The validity of this concept is apparent in the correlation of Fig. 6 between NBMPR-binding site density and araC influx rate at 1- μ M concentration of nucleoside, the latter concentration being chosen to approximate the serum levels achieved during araC infusion to leukemic patients (30). Recent genetic evidence also indicates a link between nucleoside transport rate and the number of NBMPR binding sites. Nucleoside transport in sheep erythrocytes is a polymorphic character under the control of two allelic genes (40). Some sheep have erythrocytes with nucleoside transport capability as well as specific NBMPR-binding sites, whereas such sites were absent from other sheep whose erythrocytes were impermeable to

nucleosides (16). Further evidence linking the number of NBMPR-binding sites with nucleoside transport rate comes from a study of the nucleoside-transport deficient clone (AE1) derived from the mouse T cell lymphoma, S49. These mutant cells lacked NBMPR-specific binding sites and also failed to transport a variety of nucleosides.⁵ Thus specific binding of NBMPR occurred only at functional nucleoside transporter sites.

Different leukemias vary in their responsiveness to chemotherapy with drug combinations containing araC. Indeed this drug appears to have little effect in the initial therapy of acute lymphoblastic leukemia (41–43) although it may be included in the therapy of patients who relapse (44–46). Our results show that lymphoblasts have both a lower araC influx and fewer NBMPR binding sites than myeloblasts and this lower transport capacity may be one of the several factors that contributes to the poor response of ALL to nucleoside antimetabolites. It has been recently reported that cultures of T cell lymphoblasts were more sensitive to the cytotoxic effects of araC than either null cell or B cell lymphoblasts (47). In the present study two lymphoblast preparations were from patients with T cell ALL and are shown by the open circles in Figs. 2 and 5. No differences in either NBMPR binding or araC influx were observed between these T cell lymphoblasts and the four other preparations with null cell typing.

One aim of this study was to find whether araC influx relates to the clinical response of acute nonlymphoblastic leukemia to araC. A wide variation in transport capacity of blasts from various patients with AML or AUL is evident both in Fig. 2 and 5. 15 of these patients were treated with chemotherapy and 8 entered complete remission while 7 failed therapy. The results in Fig. 7 show that three patients with a low araC influx into blasts (<0.4 pmol/10⁷ cells per min) or few NBMPR binding sites (<3,000/blasts) were among the treatment failures. Remission induction with two or three cycles of combination therapy containing araC was ineffective in eliminating either the peripheral or bone marrow blasts from these three patients, all of whom were typed as AUL by cytogenchemistry. It should be cautioned that araC transport parameters were measured on blasts from peripheral blood, while the clinical response of patients is determined by the effect of araC on blasts in the marrow. Intramedullary blasts differ from circulating blasts in having a much higher tritiated thymidine labeling index (37, 38); and greater mitotic activity of marrow blasts may confer higher transport capacity on these cells (see above). Nevertheless, peripheral blood blasts

⁵ Kolassa N., Y. Vehara, C. E. Cass, D. W. Martin, and A. R. P. Paterson. Manuscript in preparation.

that are easy to obtain and purify may still yield data of predictive value.

Is the influx rate of araC measured over 30 s relevant to the cytotoxic effect of this drug given by constant infusion for 7 d? Clearly much depends on whether the araC that enters the cell is immediately converted to its active metabolite, araCTP. An analysis of nucleoside uptake in fibroblasts has shown that when membrane transport of high capacity and low affinity is followed by a trapping step (kinase) of low capacity and high affinity then at very low nucleoside concentrations, much of the solute that enters is trapped immediately (15). Indeed, Ho et al. (48) found negligible amounts of free intracellular araC when leukemic blasts and normal leukocytes were incubated in vitro with low concentrations of araC (0.4–4 μ M). Thus membrane transport of araC at the concentrations achieved therapeutically in plasma (0.3–1.1 μ M) could be one rate-limiting step for the formation of araCTP in the leukemic blasts possessing low transport capacity. Support for this possibility comes from “protection” experiments. Animals can be protected against the lethal effect of cytotoxic nucleosides by NBMPR, a transport inhibitor whose sole action is to prevent the entry of nucleoside into the cell (49). Moreover the inhibitory effect of araC on growth of cultured leukemic cells can be reversed by NBMPR or by dipyrindamole, which is another inhibitor of nucleoside transport (50, 51). Cultured lymphoid cells in which nucleoside transport is impaired by mutation can also survive when exposed to potentially lethal doses of cytotoxic nucleosides (52). Clearly in all these situations a low rate of araC transport confers protection against the cytotoxic effect of this drug. Rustum and Preisler (53) have recently reported that in some 15% of patients with acute nonlymphoblastic leukemia, the bone marrow blasts could not convert extracellular araC to intracellular araCTP. It is not known whether this failure of leukemic cells to metabolize araC is related to low membrane transport of the nucleoside such as seen in some of our patients.

Although a low araC influx may predict a poor response to drug therapy, the converse is not necessarily true. Four of the 10 patients whose blasts gave an araC influx >1.0 pmol/ 10^7 cells per min failed to achieve a complete remission although two of the four failures did achieve partial remission. It should be recognized that the hazards of combination chemotherapy and its associated cytopenias make complete remission of the patient an imprecise measure of the cytotoxic effect of araC on blasts. This is illustrated by the failure of two patients to achieve remission because of side effects of therapy. Patient M.F. refused further treatment after achieving partial remission because of intense nausea from araC while patient V.P. developed

irreversible nephrotoxicity during gentamycin therapy for infection. Thus, a larger series of patients will be needed to find if a high araC influx predicts for clinical response to the drug. Nevertheless it seems clear that tests such as araC retention by blasts (1) or measurement of araC transport rates such as in this study will have value in predicting a poor response of certain patients to chemotherapy.

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