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SUBSTRATE SPECIFICITY OF A HIGH-AFFINITY, MONOVALENT CATION-DEPENDENT AMINO ACID CARRIER

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SUMMARY

A monovalent cation-dependent amino acid carrier was characterized in murine L1210 leukemia cells. This carrier differs from previously described ion-dependent carriers in its high-affinity for amino acids such as leucine and phenylalanine and in its tolerance of substitution of lithium and potassium for sodium ions.

INTRODUCTION

Cultured L1210 murine leukemia cells are refractory to the antitumor drug melphalan (L-phenylalanine mustard) in tissue culture medium containing amino acids (1). Investigation of the effects of single amino acids upon melphalan cytotoxicity indicated that the L-isomers of leucine and glutamine were primarily responsible for the reduced cytotoxicity (1), and that reduction in cytotoxicity was correlated with reduction in drug uptake (2). During the course of this work, it became apparent that 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), a substrate specific for the leucine-preferring transport system in the Ehrlich ascites tumor cell (3-5), was considerably less effective than leucine in reducing melphalan transport and cytotoxicity in medium containing sodium (6). This led to the observation that melphalan transport is mediated by two amino acid carriers, one sodium-independent and another sodium-dependent (6). When sodium was replaced by choline, the sodium-independent carrier was operative and melphalan transport was completely blocked by L-leucine or BCH. When the sodium-dependent carrier was operative, melphalan transport was insensitive to BCH as well as to α-aminoisobutyric acid (AIB), an alanine (A) system

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model substrate in the Ehrlich ascites tumor cell (7). The two carrier mechanism for melphalan transport has been extended to two other tumor cell types, the LPC-1 plasmacytoma (8) and the L5178Y lymphoblast (9) by Goldenberg and his colleagues. As a result of these observations, the present study was undertaken to determine the characteristics of the BCH-insensitive amino acid carrier with emphasis on its affinity for various amino acids and its requirement for monovalent cations.

MATERIALS AND METHODS

Materials. Fetal calf serum was purchased from Flow Laboratories, Rockville, Maryland and RPMI-1630 medium was supplied by the NIH Media Unit. L-Leucine, L-phenylalanine, L-alanine, L-serine and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Calbiochem, San Diego, California. α-Aminoisobutyric acid was purchased from the Sigma Chemical Co., St. Louis, Missouri and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid was obtained from New England Nuclear Corp., Boston, Massachusetts. The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°, viscosity 70 centistokes at 25°) was obtained from the Harwick Chemical Corp., Cambridge, Massachusetts. L-[4,5-^{3}H]Leucine (56.9 Ci/mmole), L-[2,6-^{3}H] phenylalanine (55 Ci/mmole), L-[2,3-^{3}H]alanine (16.1 Ci/mmole), L-[^{3}H(G)]-serine (1.8 Ci/mmole) and [carboxyl-^{14}C]inulin (1.55 mCi/g) were purchased from the New England Nuclear Corp., Boston, Massachusetts. Melphalan (11 mCi/mmole), labelled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute, Menlo Park, California. Radiochemical purity was 97% as determined by thin layer chromatography on silica gel in n-butanol-acetic acid-water (7:2:1). Labelled melphalan solutions were prepared daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid. Further dilutions were made in aqueous medium immediately prior to use in order to minimize hydrolysis of the drug.

Methods. All experiments were performed with excess BCH (2 mM) and AIB (50 mM) to block that part of melphalan uptake (50%) which occurs by the sodium-independent, BCH-sensitive L system (6) and amino acid uptake which occurs by systems L and A as described by Christensen (7). A BCH concentration of 2 mM was chosen to ensure saturation of the carrier which exhibits an apparent K_m for BCH transport of 15 μM (6). These experimental conditions allowed examination of the substrate specificity of the melphalan carrier which exhibits sodium dependence and is insensitive to BCH (6).

Various transport media were prepared in which a single monovalent cation was present either as sodium, lithium or potassium. A basal medium which contained in g/l $CaCl_2.2H_2O$ (0.1), $MgCl_2.6H_2O$ (0.1), HEPES (5.96) was supplemented with NaCl (7.3), LiCl (5.3) or with KCl (9.32) and used for experiments in which the effect of substitution of monovalent cations on amino acid and melphalan uptake was examined. The final pH of the transport medium was adjusted to 7.4 with NaOH, LiOH or KOH respectively.

The conditions used for maintenance of murine L1210 leukemia cells have been described elsewhere (1,2,6). Briefly, cells were grown in RPMI-1630 medium supplemented with 20% heat-inactivated fetal calf serum. They were harvested at the logarithmic phase of growth, washed 3 times

in transport medium supplemented with 0.1% glucose, and suspended in it at 2.0 x 10^6 cells/ml. The uptake of leucine, phenylalanine, alanine, serine or melphalan, was initiated by addition of labelled substrate to the cells at 37° to yield a cell density of 1.0 x 10^6 cells/ml. Transport of amino acids and melphalan was terminated by centrifugation of the cells through silicone oil at 12,000 x g for 1.0 minute in an Eppendorf centrifuge. Tips containing the cell pellets were removed and the pellets were solubilized in 0.2N NaOH for 3 hours at 37°. The samples were then neutralized with acetic acid and counted on a Beckman liquid scintillation counter. Data are corrected for trapped extracellular label with inulin as a marker and for a rapid adsorption component which was estimated by incubation of cells with label for 10 seconds prior to centrifugation through silicone oil.

RESULTS AND DISCUSSION

Murine L1210 leukemia cells in culture demonstrated an initial velocity of leucine transport that was 10-15% dependent upon sodium ions whereas melphalan transport was 50% dependent upon sodium ions (6). These studies suggested that this sodium-dependent portion of melphalan transport most probably occurred via the same carrier responsible for the sodium-dependent component of leucine transport. This conclusion was supported by the observation that leucine completely reduced the initial velocity of both the sodium-dependent and sodium-independent components of melphalan transport and contrasted with those results obtained with BCH, the synthetic L system substrate (3-5), which reduced only the sodium-independent component of melphalan transport.

As can be seen in Table 1, the BCH-insensitive carrier exhibits high-affinity for leucine (apparent K_m = 5-10 μM), phenylalanine (apparent K_m = 10-15 μM), and melphalan (apparent K_m = 20-25 μM). An examination of the reactivity of other naturally occurring amino acids with this carrier failed to reveal any amino acids which had a lower K_m for it than leucine (data not shown).

Two sodium-dependent amino acid transport systems, designated A and ASC, have been described by Christensen for the uptake of neutral amino acids (10). Since, in the present study, system A is essentially blocked with excess AIB, experimental conditions were sought which would determine whether the high-affinity, BCH-insensitive leucine carrier was, in fact,

Table 1. REACTIVITY OF SELECTED AMINO ACIDS WITH THE TRANSPORT CARRIER WHICH IS INSENSITIVE TO 2-AMINOBICYCLO[2,2,1]HEPTANE-2-CARBOXYLIC ACID AND α-AMINOISOBUTYRIC ACID

AMINO ACID	CARRIER-MEDIATED K_m	CARRIER-MEDIATED V_{max}	SODIUM	LITHIUM	POTASSIUM
	μM	pmoles/min/10^6 cells	V_i, pmoles/min/10^6 cells		
L-Leucine	5-10	4.3	3.6	3.0	3.0
L-Phenylalanine	10-15	4.6	4.0	3.5	4.0
Melphalan	20-25	40	27	28	20
L-Alanine	1500-2000	2000	1400	1200	2900

Murine L1210 leukemia cells, prepared as described in Methods, were incubated with radiolabelled substrate at a cell concentration of 1.0 x 10^6 cells/ml in appropriate transport medium containing 2 mM BCH and 50 mM AIB to block melphalan uptake by system L and amino acid uptake by systems L and A respectively. Transport of alanine and phenylalanine was terminated at 1.0 minute, leucine at 1.5 minutes and melphalan at 3.0 minutes by centrifugation of 1 ml aliquots (1.0 x 10^6 cells) through silicone oil at 12,000 x g for 1 minute. Tips containing the cell pellets were processed as described in Methods. Experiments from which carrier-mediated Michaelis constants were derived were performed in LiCl transport medium to differentiate this amino acid carrier from system ASC (11). The Michaelis constants were estimated according to the method of Hanes (12,13). The effect of ion substitution upon the initial velocity (V_i) of transport of the amino acids and melphalan was performed at substrate concentrations equivalent to the carrier-mediated K_m:L-Leucine (10 μM), L-phenylalanine (15 μM), melphalan (22 μM) and L-alanine (2 mM). The values for the initial velocity of uptake at the K_m concentrations of these amino acids and melphalan are slightly higher than the expected values which would be 50% of V_{max}. This may be attributed to the fact that the values of V_{max} are carrier-mediated constants while those of V_i represent total uptake by both carrier and passive diffusion.

Table 2. INHIBITION KINETICS OF THE HIGH-AFFINITY MONOVALENT CATION-DEPENDENT LEUCINE CARRIER

AMINO ACID AS SUBSTRATE	AMINO ACID AS INHIBITOR OF TRANSPORT		
	L-LEUCINE	L-ALANINE	L-SERINE
L-LEUCINE	K_m = 5-10 μM	K_i = 2.0-2.5 mM	K_i = 2.5-3.0 mM
L-ALANINE	K_i = 2.0-2.5 mM	K_m = 1.5-2.0 mM	Not Determined
L-SERINE	K_i = 1.0-1.5 mM	Not Determined	K_m = 2.0-3.0 mM

Experimental conditions were those described in the legend to Table 1. All incubations were performed in LiCl transport medium containing 2 mM BCH and 50 mM AIB. Inhibition constants were derived from Dixon plots (14).

the carrier for system ASC. The observations of Thomas and Christensen (11) that the ASC amino acid transport carrier will not tolerate substitution of lithium or potassium for sodium ions suggested that this specificity might be utilized as a criterion. As can be seen in Table 1, substitution of lithium for sodium ions has little effect on the initial velocity of transport of these amino acids and melphalan. It is apparent that potassium can also substitute for sodium ions and, in the case of alanine, potassium appears to stimulate its uptake.

Inhibition analysis of the BCH-insensitive, leucine-preferring carrier indicated that the affinity of alanine and serine for the carrier is substantially less than that exhibited by it for leucine (Table 2). This conclusion is supported by the observation that the apparent inhibition constants of alanine and serine for inhibition of leucine transport are in the range of 2.0-2.5 mM, values which approximate the apparent K_m values of these amino acids. These results also indicate that leucine is a weak inhibitor of alanine and serine transport, and suggest that the uptake of these latter two amino acids is mediated through an additional agency.

The results described in the present communication indicate that the amino acid carrier which has been identified in murine L1210 leukemia cells (6), LPC-1 plasmacytoma cells (8), and L5178Y lymphoblasts (9)

is a new, previously uncharacterized carrier which exhibits high-affinity for leucine, phenylalanine and melphalan. It differs from the ASC amino acid transport carrier in its tolerance of substitution of lithium and potassium for sodium ions.

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