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Amino Acid Requirements *in Vitro* of Human Leukemic Cells[1]

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SUMMARY

Asparaginase-induced asparagine deficiency is associated with antileukemic effects in animals and man. Amino acid requirements *in vitro* of fresh human leukemic cells were studied by means of uptake of uridine-^{3}H, to seek other amino acids which might be critical for cellular integrity. Asparagine was unique in that addition of the amino acid resulted in distinct increase of uptake in both of two patients' acute lymphocytic leukemia cells to the level seen in 6C3HED mouse lymphoma. Asparagine requirement by other leukemic cells was inconsistent, and only four of 12 reached the level seen in acute lymphocytic leukemia. No other amino acid studied substituted for asparagine.

Omission of asparagine, however, did not lower the uptake to the levels seen upon omission of cysteine, glutamine, histidine, or tyrosine, in either acute lymphocytic or acute myelocytic leukemia. Since omission of serine, glycine, proline, or alanine had little influence on uridine uptake, they are unlikely targets for depression by enzymatic destruction or for competitive analog as a nutritional approach to chemotherapy. Depression of cysteine, glutamine histidine, or tyrosine, all considered to be nonessential amino acids for whole-animal nutrition, makes these amino acids reasonable targets for enzymatic destruction or chemotherapeutic antagonism because of their apparent initial role in leukemic cells in these short-term studies *in vitro*. Partial but consistent reduction of uptake by omission of arginine in acute myelocytic leukemia cells may also warrant further exploration.

INTRODUCTION

The ability of *Escherichia coli* asparaginase to produce remissions in certain leukemias in man has now been well established (2, 11, 17). Further studies (6) on the mechanism of action of the enzyme revealed that asparaginase-sensitive leukemic cells had a low asparagine-synthesizing system and that these cells were unable to react with an increase in asparagine synthetase after exposure to asparaginase.

This specific requirement of some human leukemic cells for the nonessential amino acid, asparagine, has opened the question of whether any other amino acid occupies a similar role in man. Thus, Regan *et al.* (12) studied the incorporation of precursors of DNA, RNA, and protein by normal bone marrow and CML[3] cells in a medium devoid of some of the nonessential amino acids. They reported that normal marrow and CML cells require serine. By studying changes in amino acid levels in asparaginase-resistant and -sensitive mouse tumors, Ryan and Dworak (15) showed that glycine depression may also be involved during asparaginase treatment. Roberts *et al.* (13) recently reported that high doses of glutaminase inhibited Ehrlich ascites carcinoma in mice.

Because of these suggestions, we undertook a study, in a variety of human leukemic cells, of the requirements for amino acids other than the 8 known to be essential for whole-animal nutrition. The therapeutic implications of such requirements would allow attempts to produce other amino acid deficiencies in man. This paper reports studies of fresh human leukemic cells and their *in vitro* requirement of nonessential amino acids for uridine incorporation into the acid-insoluble fraction.

MATERIALS AND METHODS

White cells from 5 patients, 2 with ALL and 3 with AML, were studied for their *in vitro* requirements for 10 nonessential amino acids. With an understanding of the general pattern of amino acid requirements, we studied cells from an additional 9 patients (4 with AML, 2 with CML, 2 with BCML, and 1 with LsaL) for their relative requirements of asparagine and serine. In 1 of these patients, the requirement of glycine was also studied. Three of 7 patients with AML, 1 of 2 with CML, and all patients with ALL and BCML had previously been treated. At the time of study, all patients were in frank relapse and were on no chemotherapy. No patients were previously treated with asparaginase. The 6C3HED lymphoma and its characteristics have previously been described (11).

The sources of chemicals used are as follows: glycine, Fisher Scientific Co., Pittsburgh, Pa.; histidine monohydrochloride, General Biochemicals, Inc., Chagrin Falls, Ohio; nicotinamide and riboflavin, Eastman Organic Chemicals, Rochester, N. Y.; penicillin G sodium, The Upjohn Co., Kalamazoo, Mich.; streptomycin sulfate, Eli Lilly and Co., Indianapolis, Ind.; Ur-^{3}H (specific activity, 30 Ci/mmole), Schwarz BioResearch, Inc., Orangeburg, N.Y. The remaining amino acids and vitamins used were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All amino acids used were L isomers.

BEHM (3) was used. This contained the following amino

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[3] The abbreviations used are: CML, chronic myelocytic leukemia; ALL, acute lymphocytic leukemia; AML, acute myelocytic leukemia; BCML, blastic phase of chronic myelocytic leukemia; LsaL, lymphosarcoma cell leukemia; Ur-^{3}H, uridine-^{3}H; BEHM, Eagle's minimum essential medium with Hanks' balanced salt solution.

acids (mM): arginine, 0.6; cysteine, 0.1; glutamine, 2.0; histidine, 0.2; isoleucine, 0.4; leucine, 0.4; lysine, 0.4; methionine, 0.1; phenylalanine, 0.2; threonine, 0.4; tryptophan, 0.05; tyrosine, 0.2; valine, 0.4. The Hanks' balanced salt solution contained (mg/liter): $CaCl_2 \cdot 2H_2O$, 186; KCl, 400; KH_2PO_4, 60; $MgCl_2 \cdot 6H_2O$, 100; $MgSO_4 \cdot 7H_2O$, 100; NaCl, 8000; $NaHCO_3$, 350; $Na_2HPO_4 \cdot 7H_2O$, 90; dextrose, 1000; phenol red, 20. The solution contained the following vitamins (mg/liter): biotin, 1; choline, 1; folic acid, 1; inositol, 2; nicotinamide, 1; pantothenate, 1; pyridoxal, 1; riboflavin, 0.1; thiamine, 1. Similar media, each lacking 1 or all of 5 amino acids that are nonessential for whole animals were individually made: These were BEHM's minus arginine, histidine, cysteine, tyrosine, glutamine, or all 5 amino acids. Media were also made that contained each or all of the following 5 additional amino acids that are known not to be required in mammalian cell culture or whole animals: BEHM's plus asparagine, glycine, alanine, serine, proline, or all 5 of these amino acids. The final concentrations of 2 added amino acids, asparagine, 0.38 mM (10, 11), and glycine, 0.4 mM (8), were chosen from previous experience. Serine (0.5 mM) was chosen because 5.0 mM serine did not change the rate of uptake in 8 preliminary experiments. The concentration of proline, (0.5 mM) is about 3 times higher than that used in Roswell Park Memorial Institute 1640 media (9) but is about one-half that used by Regan *et al.* (12). Alanine, 0.5 mM, was arbitrarily chosen from the general levels of concentration of other amino acids. Because of technical difficulties, we have not tested glutamic acid, aspartic acid, hydroxyproline, or other less common amino acids. Each medium contained final concentrations of 15% dialyzed calf serum, penicillin G sodium, 0.3 g/100 ml, and streptomycin sulfate 0.5 g/100 ml. The pH of the media was adjusted to 7.2 and the media were sterilized by filtration (Nalgene Labware Div., Nalge Co., Rochester, N. Y.). Ur-^{3}H (final activity, 1 μCi/ml; final concentration, 33 nM) was added to the 13 different media just before each experiment.

Methods used for the *in vitro* amino acid requirement by leukemic cells were essentially similar to those described previously (11). Briefly, the peripheral blood was collected from leukemic patients in a heparin-wet syringe, and white cells were obtained by differential centrifugation. The white cells were washed twice with BEHM minus 5 amino acids and were added to the 13 different media containing Ur-^{3}H, with a final leukemic cell concentration of 10^6/ml. In acute leukemias, peripheral leukemic cells always comprised more than 75% of the total. For CML, the total peripheral leukocytes were considered leukemic. The contents of each tube were mixed thoroughly, divided into 5 2-ml aliquots in plastic tubes (Falcon Plastics. Los Angeles, Calif.), and incubated in a shaking water bath in 5% CO_2 in air at 37°. The white cells were not cooled (9) during the preparation period of less than 2 hr. The viability of separated white cells at the start of incubation was between 97 and 100%, by trypan blue dye exclusion test. At various time intervals up to 24 hr, the incubation was interrupted by the addition of 1000 times excess unlabeled uridine for a set of tubes, and incorporation of Ur-^{3}H was measured in a Packard Model 3375 liquid scintillation spectrometer after precipitation of the sample with cold 5% trichloroacetic acid on a Millipore filter disc (pore size, 0.45 μ). The 6C3HED mouse lymphoma cells were similarly analyzed, except for a final concentration of 6×10^5 cells/ml. Percentage of incorporation was calculated by the area under the curve for uptake at several time points up to 24 hr, with each media divided by that control area under the curve for uptake where the cells were incubated with BEHM only.

RESULTS

Amino acid requirements *in vitro* of 6C3HED lymphoma and human leukemia cells are illustrated in Tables 1 and 2. In the 2 human ALL cell isolates studied, levels of uptake could be divided into 3 distinct groups: an increased uptake group (BEHM plus 5 amino acids or asparagine), where 150% or more basal uptake occurred; a basal uptake group (BEHM plus serine, glycine, proline, or alanine or minus arginine), where uptake was essentially the basal 100% level of BEHM alone; and an inhibited uptake group (BEHM minus cysteine, glutamine, histidine, or tyrosine, or minus all 5 amino acids), where uptake was markedly low. An example of uptake curves in a patient with ALL (K. V.) is shown in Chart 1. The patterns of amino acid requirement of these cells were similar

Table 1

Relative Ur-^{3}H incorporation in 24 hr into leukemic cells as percentage of control in 13 different media

Percentage of incorporation was calculated by the area under the curve for uptake at several time points up to 24 hr, assuming the incorporation in BEHM to be unity. Leukemic cells, 10^6/ml, were incubated at 37° for 24 hr in 2 ml of medium, pH 7.2, containing BEHM with additions or subtractions of single amino acids or mixtures and Ur-^{3}H, 1 μCi/ml. Amino acid concentrations in parentheses expressed as mM (see text).

Cell source	Sex	Diagnosis	Prior treatment	BEHM + Asn (0.38)	+ Ser (0.5)	+ Gly (0.4)	+ Ala (0.5)	+ Pro (0.5)	+ Asn, Ser, Gly, Ala, and Pro	Alone	− Arg (0.6)	− Cys (0.1)	− Gln (2.0)	− His (0.2)	− Tyr (0.2)	− Arg, Cys, Gln, His, and Tyr
6C3HED				164	100	120	100	105	168	100	63	14	9	16	16	18
K. V.	M	ALL	Yes	187	87	98	94	87	206	100	94	9	6	6	6	11
M. S.	M	ALL	Yes	150	88	76	84	74	150	100	76	5	5	5	5	23
J. K.	M	AML	Yes	114	87	65	87	98	157	100	41	19	14	19	35	24
R. M.	F	AML	No	106	90	103	90	87	119	100	52	1	1	2	2	5
N. N.	F	AML	Yes	128	78	97	91	95	162	100	52	<1	<1	<1	<1	7

Table 2

Relative Ur-^{3}H incorporation into leukemic cells in different media

See legend, Table 1.

Cell source	Sex	Diagnosis	Prior treatment	BEHM + Asn, Gly, and Ser	BEHM + Asn and Ser	BEHM + Asn	BEHM + Ser	BEHM + Gly	BEHM Alone
J. G.	M	AML	No	218		194	88	88	100
B. B.	F	AML	No		156	168	122		100
J. M.	M	AML	Yes		152	137	102		100
S. M.	F	AML	No		123	123	101		100
D. L.	M	CML	No		154	146	108		100
C. P.	F	CML	Yes		141	126	126		100
T. H.	F	BCML	Yes		171	189	95		100
T. P.	F	BCML	Yes		112	115	73		100
J. S.	M	LsaL	No		112	106	100		100

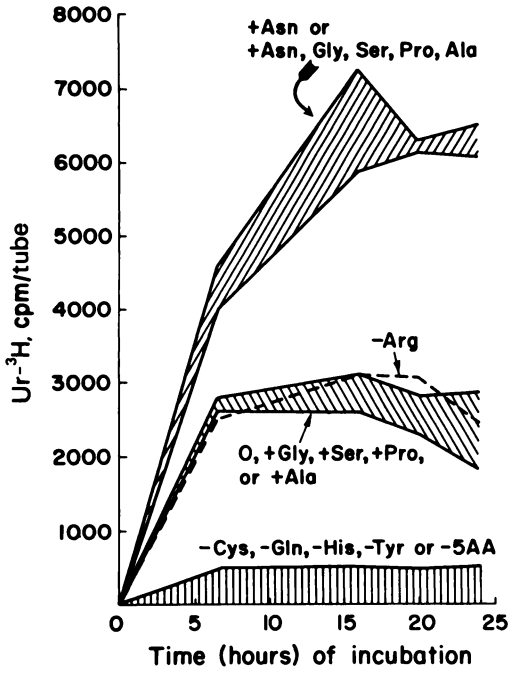

Chart 1. Ur-^{3}H incorporation by ALL cells from Patient K. V. in 13 different media under conditions described in the legend to Table 1. *0*, BEHM alone; *shaded areas*, ranges from addition or subtraction of single amino acids or mixtures as shown. *–Arg*, no difference from BEHM alone; *– 5AA*, minus cysteine, glycine, histidine, tyrosine, and arginine, simultaneously.

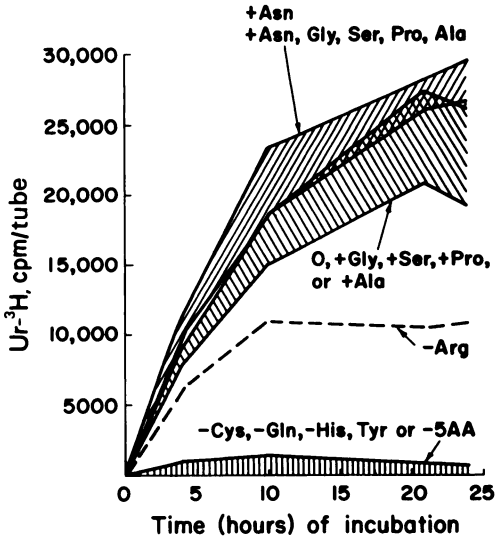

Chart 2. Ur-^{3}H incorporation by AML cells from Patient R. M. (see legend to Chart 1).

to those of 6C3HED lymphoma. The results in 6C3HED of the mouse and in these ALL cells of man may be interpreted to mean that asparagine is unique for optimal activity of ALL cells and that serine, glycine, proline, and alanine could not be substituted for it. Although lack of asparagine impairs optimal activity of ALL cells, they can still maintain reasonable RNA synthetic activity. In contrast, omission of cysteine, glutamine, histidine, or tyrosine resulted in marked decrease in cellular activity and, presumably, cell death. Arginine appears not to be required by ALL cells.

In 3 cases of AML, where 10 amino acids were studied, although uptakes of Ur-^{3}H in media supplemented with asparagine appeared to be slightly higher than basal control uptakes with BEHM alone, they never reached the levels seen in ALL or in the mouse lymphoma. An example of uptake curves in a patient with AML (R. M.) is shown in Chart 2. In 2 of 3 instances, the addition of asparagine, serine, glycine, alanine, and proline in combinations did augment Ur-^{3}H uptake. Omission of arginine resulted in consistent inhibition of incorporation to about 50% of that seen in BEHM alone. Omission of cysteine, glutamine, histidine, or tyrosine again resulted in marked decrease in cellular activity.

In 4 additional tests with cells from AML, where the effects of adding (to BEHM) either asparagine, serine (and in 1 instance, glycine), or the combination of these were compared (Table 2), uptake in 2 tests reached the level seen in ALL. Of 2 tests with cells from CML, 1 showed an approximately 50% increase in uptake with BEHM plus asparagine, and the other showed a 26% increase with BEHM plus asparagine or serine. In 1 of 2 studies with cells from BCML, a distinct requirement for asparagine but not for serine, was noted. No change in Ur-^{3}H incorporation was seen in cells from a patient with LsaL in the presence of BEHM plus asparagine, serine, or the combination.

DISCUSSION

The classic experiments of Rose *et al.* (14) have defined the normal adult human requirement for the 8 amino acids which are essential for the maintenance of nitrogen balance, *i.e.*, tryptophan, phenylalanine, lysine, threonine, valine, methionine, leucine, and isoleucine. Sugimura *et al.* (16) have shown that forced feeding to rats of synthetic diets, each lacking in 1 essential amino acid or histidine, resulted in rapid weight loss, suggesting that this was an impractical approach as a chemotherapy.

Eagle (3) has shown that growth of liver, conjunctiva, and intestine, and of carcinoma of nasopharynx (KB) and human monocytic leukemia (J111) lines, requires a minimal essential medium which includes 13 amino acids. Arginine, histidine, cysteine, tyrosine, and glutamine were added to the 8 essential amino acids, suggesting that various tissues in the whole animal can supply critical needs of these 5 amino acids from specific cell types. Lacking these producing sites, the additional 5 amino acids become essential for survival of these cell types *in vitro*. In addition to the 13 amino acids, an unusual requirement of asparagine by Walker carcinosarcoma 256 (8), of glycine by Rhesus monkey testicular cells (18) and monkey kidney cells (4), and of a combination of glycine and serine by Novikoff hepatoma cells (7) has been reported.

Because these 13 amino acids had supported long-term cultures, we chose BEHM as the starting point for our incubations. We constructed media lacking each or all of the 5 tissues culture essential amino acids and also constructed other media, adding each or all of 5 amino acids (asparagine, glycine, alanine, serine, and proline) which are known to be nonessential in usual mammalian cell culture. In the present study, it was shown that amino acid requirements of human leukemic cells followed a similar pattern, *i.e.*, the 8 essential amino acids, as well as arginine histidine, tyrine, cysteine, and glutamine. Distinct asparagine requirement was seen in the ALL cells, as well as 6C3HED cells. The incorporation of Ur-^{3}H is nearly linear for the first 15 hr in asparagine-supplemented leukemic cultures. Thereafter, the decreasing rate of incorporation may be artifactual, due to depletion of the tracer from the medium (Figs. 1 and 2). The plateau of incorporation in asparagine-deficient or nonessential amino acid-deficient media is valid, however, since adequate Ur-^{3}H was present to sustain much higher levels of incorporation. No other amino acid substituted for asparagine. The asparagine requirement is not absolute, however, since uptake in BEHM media was still much higher than that seen in media lacking cysteine, glutamine, histidine, or tyrosine. The relation between a partial requirement for asparagine by ALL cells and the definite therapeutic effect of asparagine depression by means of asparaginase is not clear. This may be 1 reason why asparaginase-produced remissions are often short and why relapse is frequent. Partial but consistent reduction of uptake by AML cells in arginine-deficient media is of interest. Although arginase is toxic in mammalian cell culture (1), arginase administration failed to produce regression of 6C3HED mouse lymphoma (5).

In the present study, we were unable to confirm the proposal that serine requirement is as important as that for asparagine (12). Most leukemic cells studied did not require serine. In 2 instances, uptake in tubes with BEHM plus serine was slightly higher, but not higher than the uptake with asparagine. In no case did the addition of glycine increase uptake more than did BEHM alone.

From the present study, it may be suggested that depression of cysteine, glutamine, histidine, or tyrosine by means of an enzyme or amino acid analog could be explored as a chemotherapeutic approach with reasonable basis to anticipate success in some acute leukemias. The relative independence of the whole mammalian organism for an exogenous supply of these 4 amino acids and the requirements of some mammalian tissues *in vitro* suggest that the spectrum of effect and toxicity will be determined in each tissue independently. This, in turn, may depend upon specific synthetase enzymes and unique tissue needs for that particular amino acid.

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