STUDIES ON THE MECHANISM OF TUMOR INHIBITION BY L-ASPARAGINASE

Effects of the Enzyme on Asparagine Levels in the Blood, Normal Tissues, and 6C3HED Lymphomas of Mice: Differences in Asparagine Formation and Utilization in Asparaginase-Sensitive and -Resistant Lymphoma Cells

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The identification of L-asparaginase as the constituent of guinea pig serum responsible for its tumor-inhibitory properties is now generally accepted (1–5). A number of other asparaginases having high substrate avidity have recently been found to inhibit tumors, notably, as first shown by Wriston and his co-workers, enzymes from *Escherichia coli* and other bacteria (6–8). Much remains to be understood, however, about the mechanism of asparaginase action in vivo and particularly the reasons for its high degree of selectivity. Normal tissues may be affected briefly, as shown by inhibition of liver regeneration (9), but rapid recovery occurs and it appears that no permanent toxicity results (1, 10). Tumors vary sharply in sensitivity to asparaginase, in some, notably certain lymphomas, severe or even complete destruction is produced, while others are unaffected (1, 3).

Earlier work has established that asparaginase-sensitive cells of Lymphoma 6C3HED are L-asparagine dependent in tissue culture, while resistant variants are asparagine independent (4). Asparagine is a constituent of most if not all proteins, and when deprived of the amino acid, protein synthesis in sensitive cells is inhibited, as has been demonstrated by Sobin and Kidd, and by others (11, 12). The present work extends from these basic findings. Its purpose has been to examine the degree of depletion of blood asparagine produced by asparaginase in vivo, and to identify metabolic characteristics of sensitive lymphoma cells which, by contrast with resistant cells and normal tissues, determine their destruction under such conditions.

Materials and Methods

Mice.—C3H or C3H/C57B1-F1 mice of either sex weighing 20–27 g were used. They were purchased from Microbiological Associates, Bethesda, Md.

Tumors.—The present studies have used, as before, cells of Lymphoma 6C3HED (desig-

nated in the figures as "S") maintained as solid subcutaneous tumors and as ascites tumors in C3H or C3H/C57B1-F1 mice. An asparaginase-resistant subline (designated "R" in the figures) was obtained at the start of the present experiments by treatment of a mouse bearing a sensitive tumor with subcurative quantities of asparaginase and repeating this over five transfer generations. The subline, as others used earlier, is completely resistant and has maintained the character continuously. In the absence of asparaginase treatment both the sensitive line and resistant sublines have the same growth rate in vivo (4).

Asparaginase.—Agouti serum L-asparaginase was obtained as described in references 8 and 13. *E. coli* asparaginase has been generously provided by the Worthington Biochemical Company, Freehold, N.J. The method of preparation is basically that of Mashburn and Wriston (6). The preparation used contained 430 units/mg, assayed by the method described previously (14). The unit of asparaginase activity is that which hydrolyzes 1 μmole asparagine in 1 hr.

Collection of Blood Samples.—To collect volumes of blood of approximately 1 ml, mice are narcotized by carbon dioxide and blood drawn from the inferior vena cava into a syringe containing dried heparin. To collect blood for asparagine estimation in animals treated with agouti serum asparaginase, the narcotized mouse is placed on a board over a magnetic stirrer. Blood is withdrawen from the inferior vena cava into a 2.0 ml syringe containing a small moving magnetic stirring bar, and initially filled to the 1.0 ml mark with 0.5 mg/ml *p*-chloromecuribenzoate (Mann Research Laboratories, Inc., N.Y.) in 0.15 M NaCl. As blood enters the syringe it is immediately mixed with this solution and, as has been found in preliminary testing, the asparaginase is at least 96% inactivated. For animals treated with *E. coli* asparaginase, 5-diazo-4-oxo-L-norvaline (the generous gift of Dr. R. E. Handschumacher) at 1×10^{-3} M was used as an inhibitor. The volume of blood entering the syringe is measured, the total contents expelled into 5 ml of 1 g/100 ml picric acid, and the syringe washed out in a further 5 ml.

Preparation of Tissues for Assays.—Mice are killed by severing the neck and blood from tissues removed by blotting on gauze. After weighing, tissues are dropped into 10 ml of chilled picric acid solution contained in the 50 ml cup of a blendor, (Omnimix, Lourdes Instrument Corp. Brooklyn, N.Y.) and homogenized at 16,000 rpm. for 2 min, keeping the cup in ice.

Measurement of Asparagine and Glutamine in Tissue Samples.—Details of the method developed in our earlier work specifically for this purpose have been published (15).

In tissue culture fluid the method is not always satisfactory due to the very high concentration of glutamine present. Asparagine is therefore measured after conversion to aspartic acid. Two 5-ml aliquots of tissue culture medium are removed, 0.2 ml of dialyzed agouti serum are added to one (to convert asparagine to aspartate), an equal volume of 0.15 M sodium chloride to the other, and the mixtures are incubated at 37°C for 60 min. Protein is precipitated by saturating with picric acid, and centrifuged down. A measured volume of supernatant is applied to a 8 x 1 cm column of Dowex 50W x 8 resin (H^+) and washed through with water until the picric acid is completely removed. Amino acids are eluted in N NH_4OH, and the eluate dried in a current of air.

The amino acid residue is applied to 3 MM Whatman paper and subjected to high voltage electrophoresis (40 v/cm) in 4×10^{-2} M phosphate buffer at pH 6.5. The acidic amino acids are eluted, and 2,4,6-trinitrophenyl derivatives prepared, as described earlier (15). After ether extraction these are separated on paper by high voltage electrophoresis in 0.1 M acetic acid–pyridine buffer at pH 5.3. The 2,4,6-trinitrophenyl derivative of aspartic acid moves more rapidly than any other colored spot and is easily identified. This is eluted in $NaHCO_3$ solution (1 g/100 ml) and its concentration estimated spectrophotometrically at 340 mμ. Above 1 μg aspartic acid reproducibility is ±3%. Asparagine concentrations in the tissue culture medium are derived from differences in optical density readings between asparaginase-

and nonasparaginase-treated aliquots. Omitting the asparaginase step, this method has been used to measure aspartate concentrations in tissues homogenized in picric acid.

Experiments on Asparagine and Leucine Metabolism In Vitro.—The purpose of these experiments has been to measure the amount of free exogenous asparagine and leucine within lymphoma cells and at the same time to measure the rate of incorporation of the amino acids into the cell proteins. To do this ascites cells washed in Eagle's basal medium (16) are suspended in 30 ml quantities of medium to which L-asparagine-^{14}C (Nuclear-Chicago Corp., Des Plaines, Ill.) and labeled leucine (L-leucine-^{14}C [UL] or L-leucine-4,5-H3, New England Nuclear Corp., Boston, Mass.) are added. The medium is contained in serum bottles at 37°C, gassed with 10% carbon dioxide in air, and gently agitated. At intervals 5.0 ml aliquots are removed, transferred to chilled McNaught tubes (Arthur H. Thomas Co., Philadelphia, Pa.) and the suspensions centrifuged immediately at 0°C and 1000 *g*. The packed cell volume is read after 10 min. Incubation medium is removed and the walls of the tube washed with fresh unlabeled medium. Distilled water is added to the 1.0 ml mark and the cells are broken by the three cycles of freezing and thawing. 1 ml of 30% trichloracetic acid is then added to precipitate proteins. After centrifugation the radioactivity of the supernatant is measured (0.1 ml in 2 ml ethanol and 15 ml toluene-Liquifluor) in a Tricarb liquid scintillation spectrometer (Packard Instruments, Downers Grove, Ill.). If similar readings are made on the free amino acids of the medium, the quantity of intracellular amino acid may be determined. It is necessary, however, to apply a correction for amino acid trapped in the extracellular space of the pellet and this may readily be done when the volume of the space is known. Average extracellular spaces of 21.23% of the packed cell volume for sensitive cells and 18.19% for resistant cells are found by the use of Dextran-Carboxyl-^{14}C (New England Nuclear Corp.). The protein precipitate from the lysed tumor cells is washed by the method of Siekevitz (17), dried and dissolved in NCS solubilizer (Nuclear–Chicago Corp.). Radioactivity is measured by liquid scintillation in Toluene-Liquifluor. Amino acid analyses of hydrolyzates (18) of proteins prepared as described have been made in the Beckman Model 120C instrument.

EXPERIMENTAL RESULTS

Blood Asparagine

Mouse plasma contains a low concentration of asparagine, which averages 34.3 nmoles/ml, a figure very similar to that found in the rat, 38.1 nmoles/ml (Table I). The asparagine level in the rabbit is rather higher, 54.9 nmoles/ml and appears more closely to resemble the figures reported in other species (19, 20). Estimations in the whole blood of the mouse yield consistently lower concentrations of asparagine than in the plasma, which is at least partly due to losses in the protein precipitate during the estimation. Nonetheless, particularly in experiments employing asparaginase, whole blood samples have been used; it is then possible to inactivate the enzyme as the sample is collected. As shown in Table I, blood asparagine levels in the mouse and plasma asparagine levels in the rat are remarkably stable under conditions of fasting. After 48 hr the levels in fasting animals do not differ significantly from those in animals on an unrestricted diet.

When C3H mice are treated with asparaginase, however, a marked fall in blood asparagine occurs. Results from experiments using agouti serum and *E. coli* asparaginase in doses which cause a moderate inhibition of tumor growth

are shown in Fig. 1. With agouti serum enzyme which is cleared slowly from the blood, in this experiment with a half-life of 11 hr, the blood asparagine remains depressed for at least 76 hr. With the *E. coli* enzyme, which had a half-life of 3 hr the level reaches normality at 44 hr. The minimum blood levels in both

TABLE I

Asparagine Levels in Blood and Plasma of Different Species (nmoles/ml)

Sample	Mouse	Rat	Rabbit
Normal plasma	34.3 ± 8.7 (6)	38.1 ± 9.2 (2)	54.9 ± 25.3 (8)
Normal blood	25.1 ± 1.7 (5)		
After 24 hr fast	21.8 ± 9.6 (3) (blood)	36.2 ± 8.9 (2) (plasma)	
After 48 hr fast	24.0 ± 6.0 (3) (blood)	36.5 ± 7.1 (2) (plasma)	

Figures shown in this and other tables are averages with standard deviations. Figures in parenthesis indicate the number of animals examined.

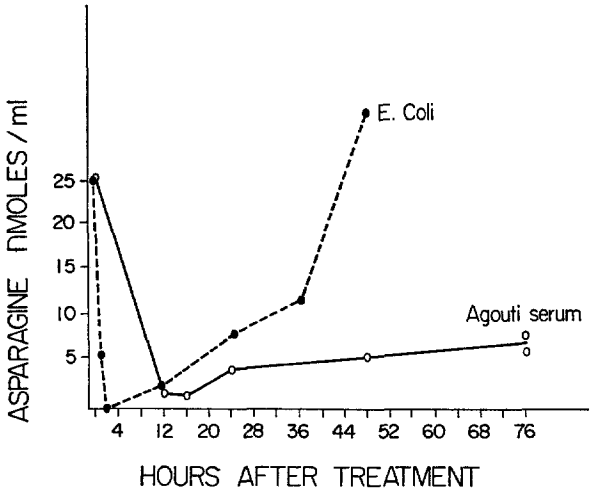

FIG. 1. Blood asparagine levels in mice injected with asparaginase. C3H mice were injected with 0.5 ml agouti serum (170 units of asparaginase) intraperitoneally, or 0.5 ml Ringer's solution containing *E. coli* asparaginase (150 units) intravenously.

Each point represents the result in an individual mouse of a group injected with the same enzyme preparation.

cases (below 1.0 nmole/ml) are much below those necessary to support the growth of 6C3HED cells in vitro, as later experiments will show. Simultaneously with changes in the blood, asparaginase causes a marked change in asparagine content of the tissues.

Asparagine Levels of Normal Mouse Tissues and of 6C3HED Lymphomas, and

the Effect of Asparaginase Treatment on These.—Normal tissues of the mouse vary considerably in asparagine content as shown in Table II. The low levels in the kidney and liver are possibly related to the fact that these two organs contain asparaginase while the others do not.[1] In solid 6C3HED lymphomas a high asparagine content is found, which is slightly more in the resistant variant than in the original asparaginase-sensitive tumor (125.1 and 143.7 nmoles/g respectively). The levels are similar to those in normal lymphoid tissue.

Asparaginase causes a marked fall in the asparagine content of normal tissues, examined 18–24 hr after treatment. In relation to the initial levels a more severe depletion occurs in the kidney than in the liver (to 24.8 and 40.3% respectively) and in the spleen as compared with the thymus (to 21.1 and 36.5%).

TABLE II

Asparagine Content of Mouse Tissues before and during Asparaginase Treatment

Tissue	Asparagine content (nmoles/g wet weight)	
	Normal	20–24 hr after asparaginase
Liver	29.3 ± 8.9 (6)	11.8 ± 5.2 (6)
Kidney	55.6 ± 9.1 (5)	13.8 ± 7.6 (5)
Spleen	124.0 ± 36.8 (9)	26.1 ± 10.5 (10)
Thymus	162.3 ± 44.2 (6)	59.2 ± 17.9 (5)
Lymphoma 6C3HED (sensitive)	125.1 ± 23.0 (10)	59.2 ± 1.5 (4)
Lymphoma 6C3HED (resistant)	143.7 ± 33.9 (8)	17.7 ± 9.6 (4)

Within 1 hr of intravenous injection of asparaginase, the asparagine content of lymphoma tissue falls to approximately 20 nmoles/g, 1/6 of the initial level (Fig. 2). Surprisingly this value is virtually the same in both sensitive and resistant forms of 6C3HED. In resistant tumors the level falls even lower during the next 20 hr, to 17.7 nmoles/g, a change which has no detectable effect on the growth rate of palpable tumors and produces no microscopic evidence of damage (4). By contrast, sensitive tumors with similar asparagine levels show marked cytotoxic effects at 6 hr, and at 20 hr, by which time the free asparagine level has risen to 58 nmoles/g, they are substantially necrotic (1). The later rise in asparagine content of sensitive tumors may be due at least in part to protein breakdown and failure of utilization of synthesized amino acids. In addition to asparagine, certain other amino acids accumulate in sensitive lymphomas at this time. For instance, in one experiment serine increased by a factor of 2.4, 20 hr after treatment, lysine by 1.9, and threonine by 1.9, from control levels of 951, 573, 1015 nmoles/g respectively.[2] Increases in the content of aspartic acid

[1] Broome, J. D. Unpublished observations.

[2] Dr. K. Woods of the New York Blood Center kindly made these measurements.

and glutamine, intermediates in asparagine biosynthesis also occur. These will be discussed more fully later.

It is thus apparently paradoxical that sensitive tumor cells die with a level of total free asparagine which is adequate for the growth of resistant cells. Further in vitro experiments, however, make it possible to provide an explanation for this finding.

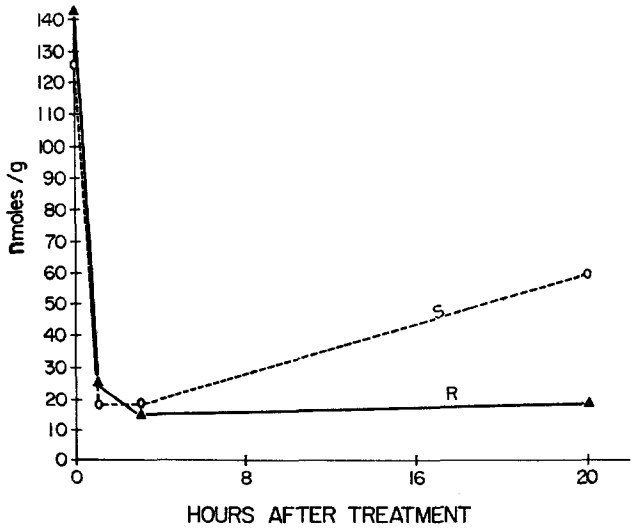

FIG. 2. Free asparagine content of 6C3HED tumors treated with L-asparaginase. Subcutaneous tumors were used 8–9 days after implantation of mice with approximately 2 million lymphoma cells. After careful blotting on gauze they weighed approximately 500 mg each. "S" indicates the asparaginase-sensitive tumor and "R" the resistant variant. For measurement of asparagine content at 1 and 3 hr, 1.0 ml agouti serum (360–420 units) was given intravenously. The number of animals used and standard deviations were: 1 hr, S-(11) ± 1.03; R-(4) ± 1.10. 3 hr, S-(4) ± 0.21; R-(4) ± 1.14. For measurements after 20 hr treatment, asparaginase was given intraperitoneally.

Utilization of Exogenous Asparagine for Protein Synthesis by Lymphoma Cells.—As has been shown in earlier work, evidence bearing on the in vivo effect of asparaginase on lymphoma cells can be obtained in tissue culture systems containing low concentrations of asparagine. It has now been possible by this means to study the effects of milder deprivation of the amino acid than can readily be demonstrated during asparaginase treatment in vivo.

Even when the medium contains an amount of asparagine which supports optimal growth (3.79×10^{-5} M) the amount of exogenous free asparagine within sensitive cells is less than in resistant (261.4 and 297.8 nmoles/1 ml cell volume respectively), and its decline with decreasing asparagine concentration in the

medium is considerably more rapid (Table III). After inhibition of protein synthesis by cycloheximide, however, both kinds of cell show the same capacity to concentrate the amino acid. These results are consistent with the view that sensitive cells utilize asparagine entering the cell from the medium at a faster rate than is the case with resistant cells. The extensive incorporation of this amino acid into cellular proteins is shown in Fig. 3. It is also clear that sensitive cells incorporate a significantly greater amount of exogenous asparagine into protein than do resistant cells.

Total protein synthesis of the lymphoma cells has been measured from the

TABLE III

Exogenous-Free Asparagine in 6C3HED Lymphoma Cells Incubated in Eagle's Medium

	Asparagine concentration in nmoles/1 ml					
						Cycloheximide
In medium	4.5	7.9	12.9	21.2	37.9	4.5
In sensitive cells	4.4	15.5	32.2	135.8	261.4	43.7
In resistant cells	20.0	50.5	77.7	162.2	297.8	39.5

The measurement of exogenous asparagine in lymphoma cells is described in the section entitled Materials and Methods; results are obtained after 10 min of incubation. In both sensitive and resistant cells maximum values for intracellular asparagine are found at this time, regardless of the external concentration used. At later times values are less reliable due to changes in the amino acid content of the medium. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) is dissolved in Eagle's medium to give a concentration of 25 mg/100 ml. Ascites cells are washed in this before beginning the incubation. During incubation incorporation of asparagine and leucine into protein is completely inhibited, but no increase in the number of cells permeable to trypan blue is found.

rate of incorporation of leucine when this amino acid is at constant concentration (2×10^{-4} M) in the medium. In sensitive cells, although the internal leucine pool remains almost constant (749–807 nmoles/ml), incorporation into protein decreases sharply when the asparagine concentration of the medium is reduced to 7.9×10^{-6} M. At 4.5×10^{-6} M, the lowest concentration obtained, leucine incorporation in resistant cells is normal (Fig. 4).

A combination of these results, by double labeling techniques, make it possible to draw certain important conclusions. It will be seen from Fig. 5 that the amount of exogenous asparagine incorporated into the cell proteins for each unit of exogenous leucine is greater in the sensitive than in the resistant cells at the highest asparagine concentration in the medium (ratios of 0.41 and 0.29 respectively). In resistant cells this ratio falls to 0.05 in an apparently linear fashion as the external asparagine concentration decreases. In sensitive cells, however, a critical change in total leucine incorporation occurs when the asparagine:leucine ratio reaches 0.23. At 12.9×10^{-6} M asparagine, total leucine in-

corporation is normal, at 7.9×10^{-6} M total incorporation falls by 35%. It is clear, therefore, that sensitive cells have only a limited ability to substitute endogenous for exogenous asparagine in protein synthesis, and that when this limit is reached, protein synthesis is inhibited.

If resistant lymphoma cells continue to make protein with the same asparagine content, it follows from the results just described that they readily replace exogenous asparagine with endogenous, as the external concentration of the amino acid decreases. Furthermore, for the result to be at all detectable the

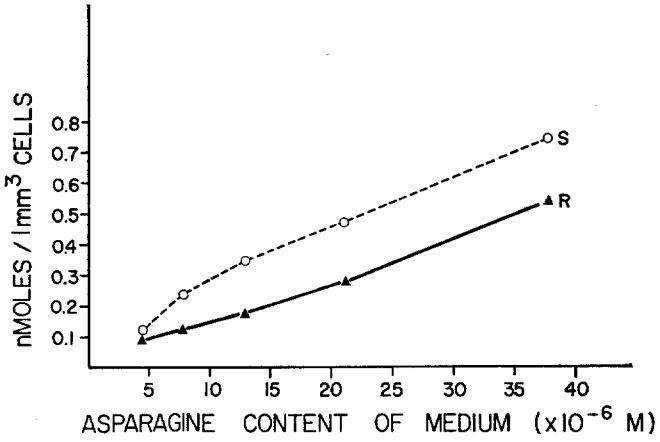

FIG. 3. Incorporation of exogenous asparagine into proteins of Lymphoma 6C3HED. Method as described earlier, measuring incorporation in 1 hr of incubation. With resistant cells (see reference 12) the rate of incorporation is linear in the 1st hr of incubation. With sensitive cells when low asparagine concentration limits utilization, the rate decreases after the first 10 min.

endogenous asparagine used in protein synthesis cannot be in equilibrium with the pool of asparagine which has entered the cells from the medium.

Asparagine is not a rare amino acid in proteins; when measured it has been found on average in approximately the same amount as aspartic acid (21), and in proteins labeled in the present experiments in amounts which are from 30–40% of their leucine content. Amino acid analyses of hydrolyzates of proteins of the resistant lymphoma cells whether grown under standard conditions in vivo or during asparaginase treatment over a whole transfer generation are, however, identical. Particularly the ratio of aspartate (which represents the sum of asparagine and aspartate in the original protein) to leucine (1.01 and 1.02 respectively) and other amino acids is the same under the two conditions. Sensitive cells not treated with asparaginase, contain a slightly higher aspartate:leucine ratio (1.11) than do resistant cells, but in general their composition is similar.

The greater utilization of endogenous asparagine for protein synthesis by resistant than by sensitive cells could be due to a higher de novo rate of synthesis or to a decrease in alternative forms of utilization of the amino acid. The latter possibility will next be examined.

Fate of Asparagine in Lymphoma Cells.—The asparagine incorporated into protein by Lymphoma 6C3HED remains within the cells; precipitates of me-

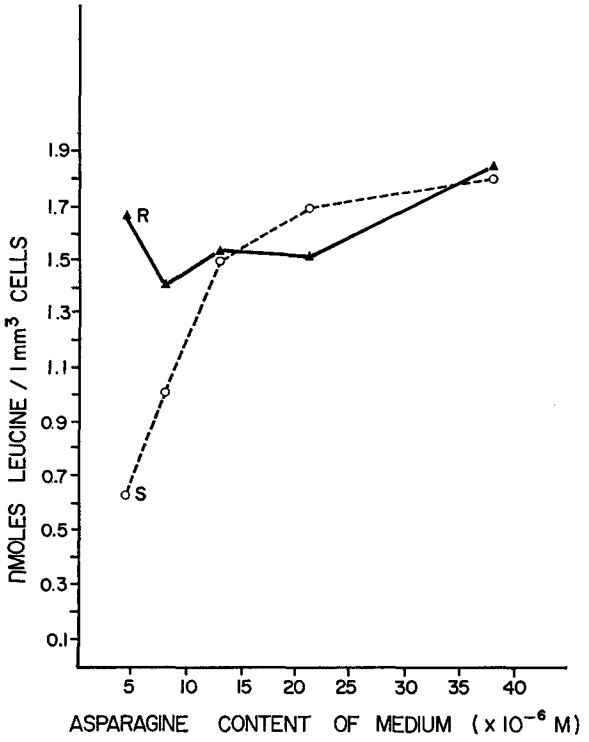

FIG. 4. Incorporation of exogenous leucine into proteins of Lymphoma 6C3HED.

dium in which lymphoma cells have been incubated with asparagine-^{14}C, show no protein-bound label. In lymphoma cell proteins, furthermore, those containing asparagine do not have a particularly rapid turn-over rate. After labeling with asparagine-^{14}C in vivo, lymphoma cells incubated for 1 hr in medium containing a high concentration of unlabeled asparagine (3×10^{-4} M) lost less than 0.5% of their label. In the cell proteins exogenous asparagine does not appear as aspartate. Proteins from both sensitive and resistant lymphoma cells incubated in asparagine-^{14}C have been hydrolyzed to amino acid and small peptide fragments by pronase (22) and these subjected to high voltage electrophoresis. At pH 1.9, approximately ¾ of the label has the mobility of

asparagine, while at pH 6.5 no detectable amount of label is found as aspartic acid or other substance with a negative charge.

No utilization of labeled asparagine for any purpose other than protein synthesis has been observed. High voltage electrophoresis at pH 6.5, 4.9, and 1.9 of protein-free lysates from cells incubated in asparagine-^{14}C show 94% of the label migrates in a fashion indistinguishable from asparagine. 6% of the label

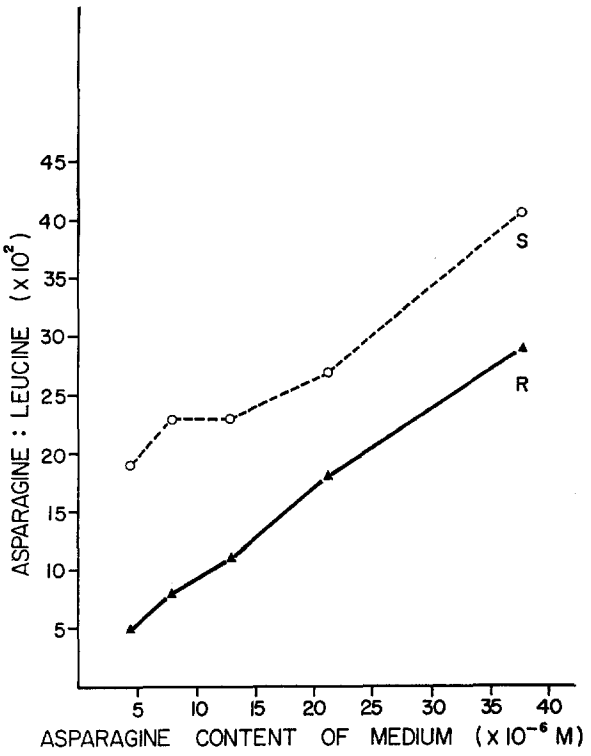

FIG. 5. Ratio of exogenous asparagine to leucine in proteins of Lymphoma 6C3HED.

is in a substance or substances with about 1/8 the mobility of asparagine at pH 1.9. Peptides migrate at this rate. No substantial amount of asparagine is incorporated into nucleotides. When precipitates of lymphoma cells in 6% trichloracetic acid have been heated at 90°C for 15 min, which hydrolyzes more than 80% of the DNA, only 1.1% of their radioactivity has been lost to the solution. Asparagine is not metabolized to carbon dioxide. In an experiment in which cells took up 10.2 nmoles of labeled asparagine per milliliter of medium, less than 0.1% of the label was found in carbon dioxide, collected in alkali, after acidification of the medium.

These results show that in both resistant and sensitive cells utilization of any

substantial amount of asparagine is by only one pathway, namely protein synthesis. A difference between these cells, however, is found in the rate of asparagine loss to medium in which they are incubated.

Loss of Asparagine from Lymphoma Cells during Incubation In Vitro.—When incubated in Eagle's medium, resistant 6C3HED cells liberate considerably more asparagine into the medium than do sensitive cells (Table IV), by a factor of 4.4 at 1 hr and by 7.0 at 24 hr. At the later time, however, only approximately

TABLE IV

Liberation of Asparagine and Aspartate into Eagle's Medium by 6C3HED Cells

Incubation period	Type of lymphoma cell	Asparagine	Aspartic acid	Cells impermeable to trypan blue
hr		*nmoles/1 mm³ packed cells*		%
1	Sensitive	0.25 ± 0.02 (4)	0.58 ± 0.09	97 ± 1
	Resistant	1.09 ± 0.13 (3)	0.22 ± 0.05	98 ± 1
24	Sensitive	3.05 2.69 Average = 2.87	17.72 19.73 Average = 18.73	50 45
	Resistant	22.5 17.9 Average = 20.2	5.59 11.09 Average = 8.34	70 72

Cells were obtained from ascites tumors. In experiments using incubation for 1 hr, an initial 10 min was allowed for equilibration before measurements were made. In experiments using 24 hr of incubation, controls were made in which replicate cultures were subjected to 2 cycles of freezing and thawing at the beginning of the experiment to cause lysis of the lymphoma cells. No increase in asparagine or aspartic acid content of the medium was found after 24 hr of incubation.

50% of the sensitive cells appear viable, while 70% or more of the resistant cells survive.

Some of the asparagine liberated by the lymphoma cells is derived from cellular protein, which is to be expected from the relatively rapid rate of protein turn-over known to occur in mammalian cells (23). In resistant cells prelabeled with asparagine-^{14}C in vivo, 0.27 ± 0.05% of protein radioactivity was recovered in free asparagine of the medium after 1 hr of incubation. With sensitive cells 0.03 ± 0.02% was recovered. Although the asparagine content of the proteins from which this asparagine is derived is not known, estimates based on the total asparagine-aspartate content of the lymphoma cell proteins indicate that proteolysis does not account for more than $\frac{1}{10}$ of the free asparagine liberated into the medium by resistant cells, and a considerably smaller amount in the case of sensitive cells.[3] It is therefore likely that the remainder is synthesized de novo.

[3] 1 mm^3 of packed cells contains 0.116 mg protein, of which approximately 5% is asparagine-aspartate, that is 4×10^{-8} moles.

In contrast to the findings just described, considerably more aspartic acid is liberated into the medium by sensitive than by resistant cells. At 1 hr, liberation of this amino acid by the two kinds of cell differs by a factor of 2.6 and at 24 hr

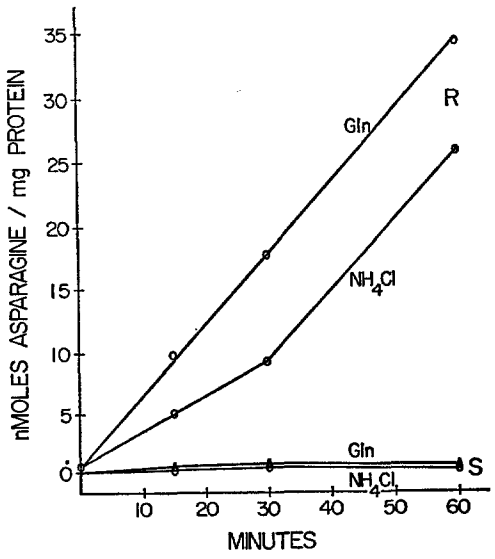

FIG. 6. Asparagine synthesis by pH 5 enzyme of cells of Lymphoma 6C3HED. Lymphoma cells were suspended in 5 volumes of 0.35 M-buffered sucrose (solution A, reference 25) and lysed by three cycles of freezing and thawing. The suspension was centrifuged at 12,000 *g* for 30 min, the supernatant diluted with 3 volumes of sucrose solution B and centrifuged at 120,000 *g* for 120 min. The supernatant from this stage was brought to pH 5.2 with dilute acetic acid and the precipitate which formed was centrifuged down at 10,000 *g* for 10 min. The precipitate was dissolved in glutathione-versene-tris buffer (pH 7.5) according to Patterson and Orr (24), and kept frozen at −20°C till use. In assays the incubation mixtures consisted of 1.0 ml 1.5×10^{-3} M L-aspartic acid with 0.25 μc L-aspartic acid $-^{14}C$ [UL] (New England Nuclear Corp.), 2×10^{-2} M L-glutamine (or 3×10^{-2} M NH_4CL), 1×10^{-2} M potassium ATP (Mann Research Laboratories, Inc.), 1×10^{-2} M magnesium chloride, 0.1 M Tris, final pH 8.0. 0.2 ml enzyme preparation was added to this. The reaction was stopped by heating aliquots in a boiling water bath for 2 min. Asparagine and aspartate were separated by high voltage electrophoresis at pH 6.5, the location of each was determined by radioautography, and radioactivity on the paper was measured by liquid scintillation counting. Protein was estimated by a modification of the Lowry method (26).

by 2.2. Further experiments show that aspartic acid is an asparagine precursor, and that considerable changes in intracellular aspartic acid levels occur in lymphoma cells treated with asparaginase in vivo.

Asparagine Biosynthesis in Cell-Free Systems.—Extracts of resistant 6C3HED cells but not of sensitive cells of this line synthesize asparagine in a system modified from that described by Patterson and Orr (24). In this a "pH 5 en-

zyme" preparation (25) is incubated with aspartic acid-^{14}C, glutamine, and ATP in the presence of magnesium ions. Labeled asparagine formed is separated from aspartic acid by high voltage electrophoresis. That the reaction product is indeed asparagine has been shown by its mobility on high voltage electrophoresis at pH 6.5 and pH 1.9, and by its conversion to aspartic acid (identified by similar means) by agouti serum asparaginase. As shown in Fig. 6, extracts from resistant cells synthesize asparagine at a constant rate for 60 min, while those from sensitive cells are completely inactive. A higher rate of synthesis has been found with two preparations from ascites tumor cells (34.8 and 35.1 nmoles asparagine/mg protein/hr) than with a preparation from solid subcutaneous tumors (10.3). Ammonium ion will substitute for glutamine; initially the reaction rate is slower but subsequently it reaches the same rate as that found using glutamine. Asparagine synthesis is inhibited to 74.3% of the maximum rate by added asparagine at 2.5×10^{-4} M, to 19.9% at 2.5×10^{-3} M, and to 7.8% at 1.0×10^{-2} M.[4]

During asparaginase treatment in vivo both sensitive and resistant 6C3HED cells show increases in substances found to be asparagine precursors, but not in their content of free α-amino nitrogen as might be expected if this were due to cell autolysis. In sensitive tumors the aspartic acid level increases to 12.0 times normal 3 hr after treatment (Fig. 7). In resistant tumors the level increases 2.9 times, and after 20 hr is still raised 1.8 times. These changes have not been found with other agents which produce cytotoxicity, for instance, in an experiment in which pairs of mice were given 58 mg/kg cyclophosphamide or 2.2 mg/kg colchicine intravenously, the average apartic acid level in lymphomas of the former was 129% of control values 4 hr after treatment, and in the latter 101%.

During asparaginase treatment glutamine levels rise in both kinds of lymphoma cell, in resistant tumors the increases (to 195% at 3 hr, and 111% at 24 hr) are again less than in the sensitive. In the latter the rise is progressive and reaches 420% at 20 hr, which may be related to the marked cytotoxic changes at this time. Such changes have been observed under different conditions by Roberts and Simonsen in several regressing tumors (28).

DISCUSSION

The results described show that in mice asparaginase treatment causes a profound fall in the normally constant blood asparagine levels. Concomitantly the

[4] The presence of asparagine synthetase is not limited to lymphomas in which resistance had developed as a result of asparaginase treatment in vivo. Preparations of pH 5 enzyme from four different mouse lymphoma lines intrinsically resistant to asparaginase synthesize asparagine at rates of 8.3–128.0 nmoles/mg protein per hour. By contrast, no synthetase activity has been found in extracts of cells of two further lines of asparaginase-sensitive lymphomas.

total free asparagine content of both normal and lymphomatous tissue decreases. But it is clear that the degree of fall bears no relation to the amount of cellular damage which occurs. The liver, for instance, at 11.8 nmoles/g has a level of asparagine lower than ever found in lymphoma tissue. Sensitive lymphomas maintain an average level of 19.4 nmoles/g 1 hr after treatment,

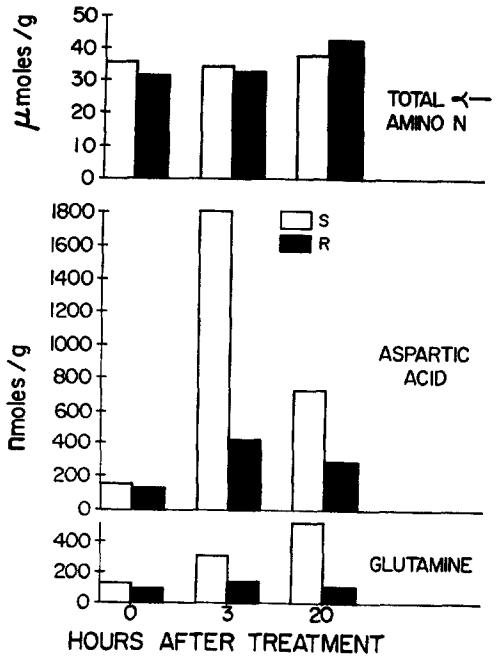

FIG. 7. Effect of L-asparaginase treatment on amino acid levels in 6C3HED tumors. Experimental animals were treated as described in Fig. 2, with 1.0 ml agouti serum (340 units). Total free α-amino nitrogen was measured by the ninhydrin reaction (27) on picric acid supernatants used for assay of asparagine and other amino acids. Results in each case represent averages of three to six experiments.

very little different from that in resistant tumors; at 3 hr the two kinds have equal levels.

That resistant 6C3HED cells are able to continue unimpaired growth under these conditions depends on several factors, first, that they have a high rate of asparagine synthesis. This is shown by their ability to substitute endogenous for exogenous asparagine in freshly formed proteins when deprived of an external source of the amino acid, and at the same time to lose substantial amounts of asparagine to the medium. Sensitive 6C3HED cells appear to have some capacity to substitute endogenous for exogenous asparagine (change of leucine:asparagine ratio from 0.41 to 0.23 with a normal rate of leucine incor-

poration into protein), and to lose more asparagine to the medium than can be accounted for by proteolysis, but in both cases this is considerably less than with resistant cells. However, no asparagine synthetic system has been demonstrated in extracts of sensitive 6C3HED cells or cells of two other asparaginase-sensitive lines. But an active system has been obtained in pH 5 enzyme preparations from asparaginase-resistant 6C3HED cells, which utilizes aspartic acid, glutamine, and ATP. Asparagine concentration, itself, may act as a rate control mechanism for the synthetase. An apparently similar asparagine synthetase has been obtained from the cells of four lymphoma lines which are intrinsically resistant to asparaginase, and from certain normal tissues. Whether the presence of this system is a consistent characteristic of asparaginase-resistant cells and its absence characteristic of asparaginase-sensitive cells remains to be determined. It is not unlikely, however, that in either kind of cell there are other routes for asparagine synthesis. Arfin has shown a different synthetic system in embryonic chick liver (29) and in bacteria others are known (30, 31).

A further factor responsible for the asparaginase resistance in 6C3HED-R lymphoma cells is their ability to conserve synthesized asparagine for protein synthesis. To be able even to detect substitution of exogenous by endogenous asparagine in resistant cells, the rate of exchange between intra- and extracellular asparagine when the amino acid is at a low level in the medium must be slower than the rate of asparagine utilization. Relative impermeability, how ever, does not explain the observation that in solid tumors in vivo, protein synthesis is strongly inhibited in sensitive cells at levels of total cell asparagine which are adequate for normal synthesis in resistant cells. This raises the possibility that there is a linkage between sites of asparagine synthesis and utilization in resistant cells, which allows synthesized asparagine to be used preferentially for protein formation before equilibration with the whole cell pool. Such a system could be of great importance for protein synthesis in tissues such as the liver and kidney which normally contain asparaginase, and in these and others during asparaginase treatment. It may also be important in vitro: Eagle and his coworkers have obtained evidence of the preferential use of endogenous over exogenous asparagine in protein formation by HeLa and other asparagine-independent cell lines (32).

In addition to inhibiting protein synthesis, it has been shown that asparaginase causes changes in the intracellular levels of other amino acids, particularly, in sensitive cells, the accumulation of aspartic acid to 12 times the normal. It has not been possible to test the isolated effect of this change: no comparable rise in intracellular aspartic acid occurs when cells are incubated in high concentrations of the amino acid (to 3.0×10^{-3} M). The role of these effects in causing cytotoxicity cannot, therefore, be excluded, and indeed it is possible that cytotoxicity from asparaginase treatment is due to a combination of separate events. Nonetheless, inhibition of protein synthesis is likely to be of

particular importance, and may affect a number of metabolic control mechanisms. Alkaline ribonuclease, for instance, is activated within 1 hr of asparaginase treatment of sensitive tumors (33). Further, it may be that inhibition of protein synthesis by asparaginase is selective so that particular essential cell proteins are no longer formed. In cell-free bacterial systems asparaginase has been shown to inhibit the formation of an asparagine-rich but not of an asparagine-poor protein (34). These later effects of asparaginase treatment are at present under investigation.

SUMMARY

L-asparaginases of agouti serum and *Escherichia coli* cause a profound lowering in the level of free asparagine in the blood of treated mice and also in the tissues. During treatment, normal tissues and resistant 6C3HED lymphomas survive unharmed with intracellular asparagine levels which are critically low for sensitive lymphomas. An explanation for this contrast between the two types of lymphoma is provided by the finding that resistant cells have not only a higher asparagine synthetic capacity than sensitive cells but appear able to utilize endogenous asparagine preferentially for protein synthesis. Cell-free extracts of resistant cells contain an asparaginase synthetase, but this is not found in preparations from sensitive cells.

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