ASPARAGINE SYNTHETASE IN ASPARAGINASE RESISTANT AND SUSCEPTIBLE MOUSE LYMPHOMAS

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Since the original discovery of a tumor inhibitory factor in guinea pig serum by Kidd (1953) and its identification as L-asparaginase by Broome (1961), this enzyme has been used to regress tumors, especially lymphomas, of the mouse, rat, dog, and man. The work of Sobin and Kidd (1966) showed that protein synthesis was the first biochemical system to be inhibited by asparaginase treatment of a susceptible tumor. Broome and Schwartz (1967) reported that resistant tumors have a greater endogenous supply of asparagine. Furthermore, Patterson and Orr (1967) found that an asparaginase resistant form of the Jensen sarcoma had a greater capacity for asparagine synthesis than either a susceptible line or normal liver. The present studies show that asparaginase resistant lymphomas have higher levels of asparagine synthetase (ASase) than either susceptible tumors or normal tissue. In addition, resistant tumors responded to asparaginase treatment with large increases in ASase levels, a moderate response was noted for normal tissue, and a susceptible tumor gave only a transient response.

Materials and Methods

6C3HED-S (asparaginase susceptible) and 6C3HED-R (asparaginase resistant, kindly made available by Dr. Louise Mashburn) sublines of the Gardner lymphosarcoma have been carried in ascites form by serial transplant in C3H/HE mice. The P388 leukemia, obtained from Dr. James Belli, was carried in DBA/2 mice. Tissue from AKR mice was from pre-leukemic animals and from those with spontaneous leukemia.

E. coli asparaginase used in these studies was from Worthington Biochemical Corp. It was assayed in a 2.0 ml mixture which was 0.01 M asparagine and 0.05 M Tris chloride, pH 8.6. Liberated NH_3 was measured by nesslerization. For ASase assays, cells suspended in 0.9% NaCl were disrupted by ten cycles of freezing and thawing. After centrifuging 30 min at 10,000 rpm (Sorvall SS-34 rotor), the supernatant fluid was removed, and 0.1 - 0.2 ml containing 1-4 mg protein was used for assay. In addition to enzyme, the 1.0 ml reaction mixture contained 1.5 mM aspartate-C^{14}, 20 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM ATP, 4 mM puromycin, and 0.1 M Tris, pH 7.6. After incubating 30 min at 37°C, reaction was stopped by addition of 1.0 ml 0.8 M $HClO_4$. Cold carrier asparagine (0.5 mg) was added, and the pH was increased to between 3 and 7 with KOH. Following centrifugation, the supernate was washed through an alumina column (0.3 x 15 cm) with 10 ml water. This eluate contained the asparagine free of aspartate (Bessman, 1957). Aliquots were removed (0.5 ml) for determination of asparagine concentration (Rosen, 1957) and radioactivity. The latter was measured with 77% efficiency in a PPO-dimethyl POPOP solution in a 1:2 (v/v) Triton X-100:toluene mixture in a Nuclear Chicago Mark I liquid scintillation counter. The amount of asparagine synthesized was calculated from a standard carrier formula.

All enzyme activities are expressed in international units/mg protein.

Results and Discussion

To prove that radioactivity recovered in the water wash of the alumina columns was asparagine, eluate was subjected to paper electrophoresis after adding cold aspartic acid and asparagine. Electrophoresis was performed with Gelman high resolution buffer, pH 8.6; staining was with ninhydrin. Aspartate and asparagine were well separated, and all of the radioactivity coincided with the asparagine spot (Packard Radiochromatogram Scanner). If eluate was subjected to asparaginase before electrophoresis, radioactivity then coincided with aspartic acid. In assays of mouse liver, a second radioactive material appeared in the column effluent. In addition,

preparations of liver ASase contained asparaginase as well, making evaluation most difficult. The pH optimum for ASase from 6C3HED-R and P388 was 8.0 in agreement with that for the Jensen sarcoma (Patterson and Orr, 1967). Up to 75 x 10^{-4}U of ASase per assay gave a linear response to changes in enzyme concentration; at the upper level 15% of the aspartate was converted to asparagine. Deletion of ATP from the assay mixture completely inhibited asparagine formation. Without puromycin, asparagine synthesis decreased 45-60%. Although puromycin was included to prevent asparagine utilization in protein synthesis, its mechanism of action has not been studied. ASase is apparently product inhibited; with L-asparagine added to the assay mixture so as to give a 1:1 ratio with substrate L-aspartate, 32% inhibition was observed. At the same concentration D-asparagine inhibited less than 2%. ASase from two different bacterial sources proved to be much more sensitive to product inhibition (Ravel et al., 1962; Burchall et al., 1964).

As seen in Table I, no ASase was found in normal C3H mouse lymphoid tissue (spleen), and only a trace was detected in normal AKR spleen, which may be considered pre-leukemic because of the high incidence of AKR leukemia. Leukemic AKR spleen had only slightly more ASase activity. For 6C3HED-S cells the values also were quite small and were frequently zero. The asparaginase resistant tumors represented by AKR leukemic lymph node, 6C3HED-R, and P388 all had significantly higher ASase activities. After asparaginase treatment comparable tissues showed interesting increases in ASase activity. Normal lymphoid tissue gave a modestly increased specific activity while ASase levels in resistant tumors increased dramatically (up to 20 fold). For 6C3HED-S the rise was quite small and was followed for only 8 hours because it had already passed its maximum.

The time course of the increase of ASase in normal C3H spleen, 6C3HED-S, and 6C3HED-R tumor cells following asparaginase administration is given in Table II. In the resistant tumor, the specific activity of ASase doubled in

TABLE I

Effect of Asparaginase on Asparagine Synthetase of Mouse Lymphoid Cells

Tissue	Mouse Strain	Asparaginase Treatment (U/mouse)	ASase (10^{-4}U/mg prot) No Rx	24 Hrs After Rx
Normal Spleen	C3H	5.0	0	3.59
Pre-leukemic Spleen	AKR	2x2.9	0.07	1.20
Leukemic Spleen	AKR	2x2.9	0.19	1.40
Leukemic Lymph Node	AKR	2x2.9	0.52	9.80
6C3HED-S	C3H	2.3	0.05	0.57[a]
6C3HED-R	C3H	5.0	2.10	12.0
6C3HED-R	C3H	2x5.0	1.80	12.6
6C3HED-R	C3H	3x4.6	1.90	12.8
P388	DBA/2	3x5.0	1.80	7.60

a) Values are for 8 hours after asparaginase administration. ASase had already passed its maximum.

the first 4 hours, doubled again in the next 4, and continued to increase for at least 24 hours. Although increases were smaller in normal spleen, the pattern was similar with ASase again doubling between 4 and 8 hours. This ability of normal tissue to respond with an increase in ASase, which was maintained for at least 48 hours, can account for the low toxicity

TABLE II

Asparagine Synthetase Activity in Mouse Lymphoid Cells As A Function of Time Following Asparaginase Administration

Hours After Asparaginase Administration	ASase (10^{-4} U/mg Protein) Normal Spleen[a]	6C3HED-S[b]	6C3HED-R[c]
0	0	0	1.8
2	---	0.04	---
4	1.05	1.14	3.4
6	---	0.62	---
8	2.12	0.40	7.3
24	3.59	---	11.1[c]
48	3.26	---	12.6
72	---	---	12.8

a) 5.0 U asparaginase given to non-tumor bearing C3H mice

b) 2.3 U asparaginase administered

c) 5.0 U asparaginase administered at zero time and again after 24 hrs

of asparaginase. With the susceptible tumor there was a small initial rise in ASase, paralleling normal lymphoid tissue for 4 hours. The peak activity was reached at this time, however, and subsequent values were lower. The period of decreasing values was also a period of significant cell death. Morphologic examination of peritoneal washings showed that there was no influx of macrophages during the 8 hr period of observation and that the cells on which assays were performed were indeed tumor cells. These data provide a reasonable basis for tumor cell susceptibility or resistance to asparaginase treatment. Derepression probably accounts for the major part of the ASase increase; data relative to this point will be presented elsewhere.

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