Studies on the Aspartic Acid "Sparing Effect" on the Nutritional Requirement of L-Asparagine for Tumors *in Vitro* (33821)

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The basis for the current use of L-asparaginase as a therapeutic agent in the treatment of certain malignant diseases is the nutritional requirement of certain malignant cells for L-asparagine (1, 2). Those cells sensitive to the enzyme have been shown to lack the capacity to synthesize L-asparagine in quantities sufficient to support cell growth (3–5) and are thus dependent upon exogenous sources. McCoy *et al.* reported that the L-asparagine requirement of two tumor cell lines, a (3'MDAB)- induced hepatoma (6) and the Jensen sarcoma (7), was spared by high concentrations of L-aspartic acid. Broome reported evidence that suggested some sparing effects of aspartic acid on the asparagine requirements of the 6C3HED tumor cells (8). The Walker 256 carcinoma, a tumor also requiring L-asparagine, however, was reported to be unable to replace the requirement by aspartic acid (9). Subsequently, Eagle confirmed these observations and reported that the sparing effect was population dependent (10) with the Jensen sarcoma but not with the Walker 256 carcinoma. At high population densities the 3'-MDAB hepatoma was able to dispense with both amino acids. As a result of these studies it was suggested that a promotion of asparagine synthesis in the Jensen sarcoma and the 3'-MDAB hepatoma under these conditions might be analogous to the induction of glutamine synthetase by high concentrations of glutamic acid (10, 11). Cells, however, appear to preferentially utilize aspartic acid, newly synthesized from glucose and glutamine, for protein synthesis and presumably asparagine biosynthesis, rather than that provided by exogenous sources (10, 12, 13).

The recent development of an assay system for asparagine synthetase (4, 14) allowed us to investigate the possible *in vitro* "inductive effect" of high concentrations of aspartic acid on the enzyme. As a consequence of these studies, the nutritional "L-aspartic acid sparing effect" for cells in culture was reinvestigated.

Materials and Methods. Cell inocula were prepared from freshly excised Jensen sarcoma and Walker 256 carcinoma, carried as transplants in the *rectus femoris* muscle of female Holtzman rats. The cells were established for 24 hr in a 5% CO_2 incubator either in Blake bottles or T-15 flasks in triplicate in McCoy's medium 5a, which contains 0.3 m*M* L-asparagine and 0.15 m*M* L-aspartic acid (15), supplemented with 5% Sephadex-treated bovine serum. The medium was replaced with the experimental medium, which was changed every 24 hr thereafter. The cells were harvested, following a medium change, by cooling for 1 hr at 4° and counted in a Coulter model B cell counter. Cells for enzyme assay were collected by centrifugation and washed 3 times with cold Earle's solution. Asparagine synthetase was assayed by a previously described method (4, 14) modified by using the 20,000*g* supernatant for assay purposes. Protein was determined by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

Assay for L-asparagine as a contaminant of L-aspartic acid, purchased from various commercial sources, was made by the addition of 10.0 IU of *E. coli* L-asparaginase (kindly supplied by Dr. Joseph Roberts, Wadley Institutes of Molecular Medicine, Dallas, Texas) to 1 ml of 60 m*M* L-aspartic acid. Following a 30-min incubation at 37°, the ammonia released was assayed directly by the procedure of Chaney and Marbach (17).

Results. Incubation of the Walker 256 or Jensen tumor cells in medium devoid of both L-aspartic and L-asparagine resulted in an exponential decrease in the cell population during the 7-day observation period (Fig. 1). The addition of 10 m*M* L-aspartic acid to the medium, however, gave variable results.

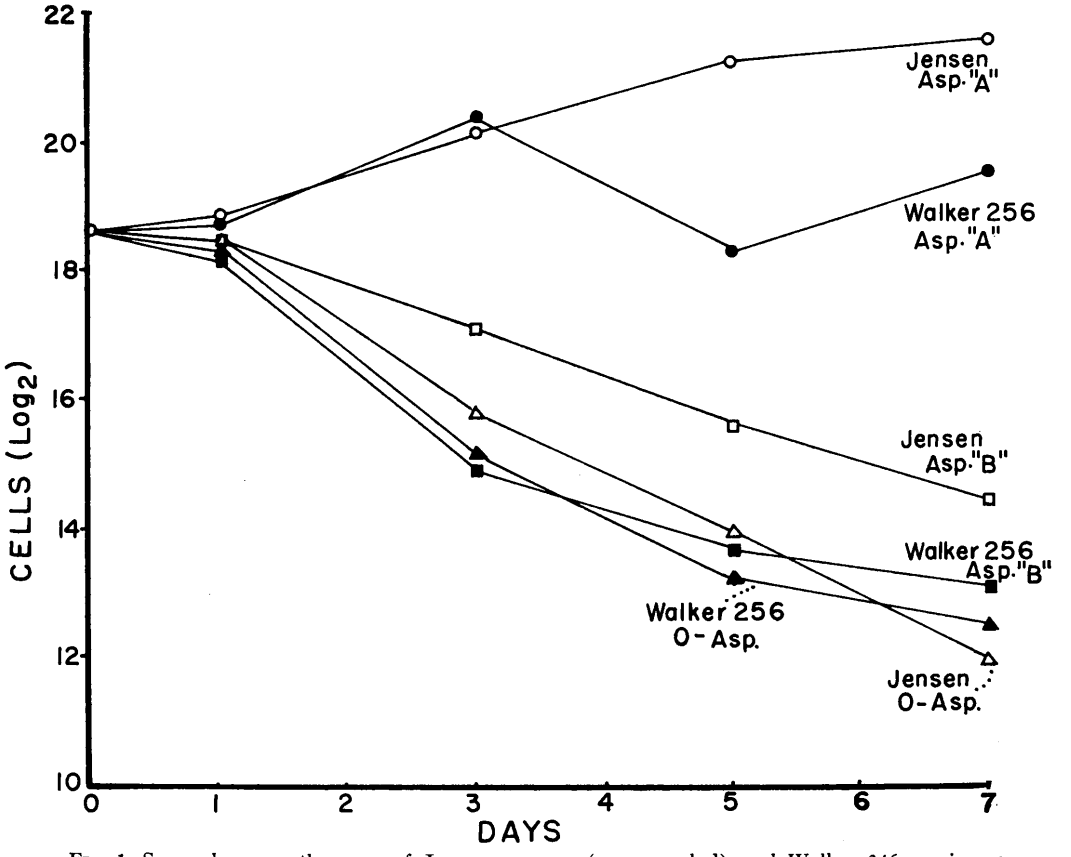

FIG. 1. Seven-day growth curve of Jensen sarcoma (open symbol) and Walker 246 carcinoma (closed symbol) in McCoy's medium 5a minus L-asparagine, (△); and the absence of L-aspartic acid, (▲); or the presence of 10 m*M* L-aspartic acid (Supplier A, contaminated with 1.5% L-asparagine, (○), (●); and Supplier B, no detectable L-asparagine, (□), (■).

Supplier "A" aspartic acid caused cell proliferation, whereas "B" aspartic acid gave essentially the same results as those obtained with no supplemental aspartic acid. Analysis of the aspartic acids used revealed a contaminant of L-asparagine (0.15 m*M*/10 m*M* L-aspartic acid) in "A" and no detectable amide in "B". The growth curves from the preceding experiments suggested a difference in the asparagine requirement of the two cell lines. Table I shows that while 0.03 m*M* will adequately support the Jensen sarcoma, the Walker showed only minimal growth at this concentration.

To determine whether a population dependency existed for growth of the cells in high concentrations of L-asparagine-free L-aspartic acid (Supplier B), inocula varying between 4.5×10^3 and 4.5×10^6 cells per T-15 flask (1.5×10^3–1.5×10^6 cells/ml) were cultured in the three media (Table II). At high inocula for the Jensen sarcoma (1.5×10^6/

TABLE I. Concentration Effect of L-Asparagine on the Growth of Jensen Sarcoma and Walker 256 Carcinoma.

Asparagine (mM)	7-Day growth[a]	
	Jensen	Walker 256
0	0[b]	0[b]
0.03	29.9	1.78
0.30	30.2	19.4

[a] Values represent cell growth where initial inoculum (1.5×10^4 cells/ml) = 1; average of triplicate flask counted in duplicate.

[b] Value <0.1.

TABLE II. "Aspartic Acid Sparing Effect" on Varying Cell Inoculum.

Cells	Medium	Cell growth[a] (inoculated cells/ml)			
		1.5×10^6	1.5×10^5	1.5×10^4	1.5×10^3
Jensen	Complete	0.71[b]	7.3	3.4	6.8
	Minus ASN and ASP	0.02	0.04	0.04	0[c]
	Minus ASN plus 10 m*M* ASP	0.01	0.11	0.10	0
	Minus ASN plus 15 m*M* ASP	0.03	0.17	0	0
	Minus ASN plus 20 m*M* *ASP*	0	0.14	0.04	0.04
Walker	Complete	—[b]	0.84[b]	6.4	6.8
	Minus ASN and ASP	0.07	0.01	0	0
	Minus ASN plus 10 m*M* ASP	0.06	0.03	0.09	0
	Minus ASN plus 15 m*M* ASP	0.02	0.02	0	0
	Minus ASN plus 20 m*M* ASP	0.03	0.02	0	0

[a] Values represent cell growth where initial inoculum equals 1; average of triplicate flasks counted in duplicate; 5-day growth.

[b] Overgrowth of flask resulting in cell sloughing.

[c] Values of 0 gave growth of <0.01.

ml) and the Walker 256 (1.5×10^6 and 1.5×10^5/ml) excessive growth in the complete medium occurred and the cells failed to adhere to the flask as a result of acid production. In contrast, the cells grown in the absence of L-asparagine and L-aspartic acid or the absence of L-asparagine plus 10, 15, or 20 m*M* L-aspartic acid showed no evidence of acid production or growth. At the same cell inocula where optimum growth was obtained in complete medium, the cells failed to grow in experimental media containing 10, 15, or 20 m*M* L-aspartic acid.

The asparagine synthetase activity of Jensen sarcoma cells grown in the presence of 10 m*M* aspartic acid "A" or "B" remained constant over the 5-day culture period (Fig. 2). The asparagine synthetase activity of cells cultured in complete medium 5a also remained constant throughout the 5-day culture period.

Discussion. The data obtained in the present studies suggest that the previously reported "L-aspartic acid sparing effect" on the asparagine requirement of certain tumor cells in culture reflected the use of L-aspartic acid containing L-asparagine as a contaminant. The addition of 10 m*M* L-aspartic acid, shown to contain 1.5% L-asparagine, to culture medium devoid of L-asparagine supported the growth of both the Jensen sarcoma and the Walker 256 carcinoma. When the two cell lines were cultured in media containing 10 m*M* unadulterated L-aspartic acid or deprived of both L-aspartic acid and L-asparagine, the population decreased 4- to 6-fold during a 7-day culture period. In both instances when 10 m*M* L-aspartic acid was

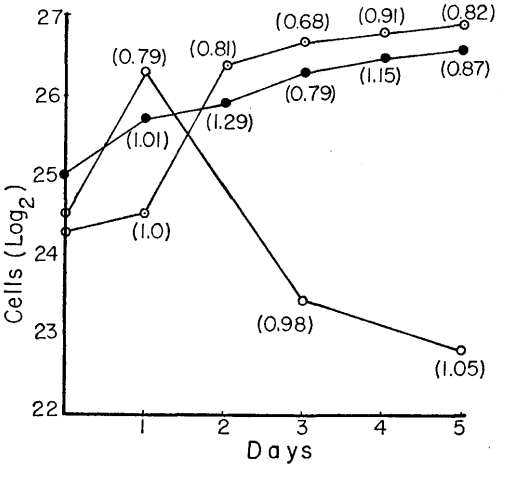

FIG. 2. Asparagine synthetase levels during the growth of the Jensen sarcoma and the effects experimental medium had. Complete medium 5a, (⊙); complete medium 5a minus asparagine plus 10 m*M* aspartic acid "A," (●); or 10 m*M* aspartic acid "B", (○). The values in parentheses represent the mmoles of asparagine synthesized/mg of protein/hr at each time period.

used, the growth of the Jensen sarcoma was less affected than the Walker 256 carcinoma. This would appear to result from a difference in the requirement of the two tumors. This could explain the anomalous results obtained by the previous investigators (9, 10) that high concentrations of aspartic acid (presumably contaminated with asparagine) would support the Jensen sarcoma but not the Walker 256 carcinoma.

Cell inocula had no apparent effect on the growth of either cell line in the presence of high concentrations of L-aspartic acid. High cell inocula (4.5×10^6 or 1.5×10^6 cells/ml) of both cell lines in complete media could not be evaluated as the result of high lactic acid production and a consequent cell sloughing. With the Walker 256, inoculum of 1.5×10^5 cells/ml produced enough lactic acid to prevent the cells from sticking and the cells sloughed within 48 hr. At the same inoculum, the Jensen sarcoma showed exponential growth. Thus a second anomaly, that of cell inoculum, especially at high densities, could explain the Walker 256 carcinoma's lack of response to high concentrations of aspartic acid adulterated with L-asparagine.

Supplying the Jensen sarcoma with sufficient L-asparagine to maintain proliferation, either as a contaminant of aspartic acid or as a supplement to the media or in the presence of 10 m*M* aspartic acid, did not alter the asparagine synthetase activity over a 5-day period. It would appear, therefore, that aspartic acid, even at high concentrations, does not induce asparagine synthetase activity.

Summary. The L-aspartic acid sparing effect of the nutritional requirement for L-asparagine for the Jensen sarcoma and the Walker 256 carcinoma was reinvestigated. The effect was found only when the aspartic acid contained L-asparagine as a contaminant. Studies on the induction of asparagine synthetase by high concentrations of aspartic acid gave negative results.

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