Response of Intracerebral Gardner Lymphoma to Guinea Pig L-Asparaginase and *Escherichia coli* L-Asparaginase.* (31422)

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(Introduced by G. M. Hass)
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The antilymphoma effect of guinea pig serum(1) appears to be related to its L-asparaginase activity(2). Protein fractions isolated from *Escherichia coli*(3) which have L-asparaginase activity are also effective against the 6C3HED mouse lymphoma. 6C3HED lymphoma cells maintained in tissue culture grow more rapidly in asparagine-rich medium. It was further shown that the 6C3HED lymphoma cells assimilate C^{14} labeled asparagine, but not aspartic acid(4).

Whole guinea pig serum which is effective against subcutaneous 6C3HED tumors was not effective in the same dose against intracerebral 6C3HED tumors(5). In one leukemic human treated with guinea pig serum L-asparaginase, a peripheral tumor regressed, but the patient expired with meningeal and cerebral lymphoma(5).

The following paper describes the effect of partially purified preparations of L-asparaginase from guinea pig serum and from *E. coli* on intracerebral 6C3HED tumors.

Materials and methods. Tumors and mice. The solid form of 6C3HED lymphosarcoma, female C3H mice from Cumberland View Farms, and C3H/An mice from Hines V.A. Hospital were used. Cells were inoculated in a constant volume of 0.03 cc of Hanks' solution into the right cerebral hemisphere through a 27 gauge needle. The method of counting cells was described previously(5). Animals were fed Purina Mouse Breeder Chow and oxytetracycline HCl (0.33 mg/cc) in the drinking water during the complete experiments.

Histology. Mice were autopsied and their tissues fixed in 10% formalin. After fixation, brains were examined by serial coronal sections, blocked in paraffin, and sectioned for routine histology. Tissues were stained with haematoxylin and eosin stain.

Growth of *E. coli* and preparation of cell-free extract. *E. coli,* wild type, was obtained from our Department of Microbiology stock and grown on Difco nutrient broth. Cultures were transferred serially to 20 liters of medium in a 12 gallon carboy and incubated at room temperature with aeration. The cells were harvested by centrifugation and washed twice with 0.05 M tris buffer pH 8.6, suspended in the same and sonicated for 10 minutes with a Branson 125 watt sonifier. Unbroken cells and cell debris were removed by centrifuging for 20 minutes at 40,000 $\times$ *g*.

Enzyme purification. *E. coli* L-asparaginase. The cell-free extract of sonicated *E. coli* cells was fractionated according to the procedure described by Mashburn and Wriston(3). Nucleic acids were removed by adding 0.05 volume of 1 M $MnCl_2$ in the cold. The sediment was removed by centrifuging 20 minutes at 40,000 $\times$ *g*. The supernatant was brought to 55% saturation with $(NH_4)_2SO_4$ and centrifuged for 20 minues at 40,000 $\times$ *g*. The supernatant was decanted and brought to 100% saturation with $(NH_4)_2SO_4$ and centrifuged for 10 minutes at 10,000 $\times$ *g*. The precipitate was dissolved in deionized water and dialyzed overnight against 3 changes of 0.05 M tris buffer of pH 8.6. It was frozen and stored at $-60°$. Assays used for L-asparaginase and protein were indentical to those reported previously(5).

Guinea pig L-asparaginase. Partially purified guinea pig L-asparaginase (PPLAFI) was prepared by the method described previously (5,6,7). It was administered in one dose per mouse *via* the intraperitoneal route (Table II).

Results. Enzymes. The specific activity (μM NH_3 liberated per hour per 1 mg protein in the presence of an excess of L-asparagine)

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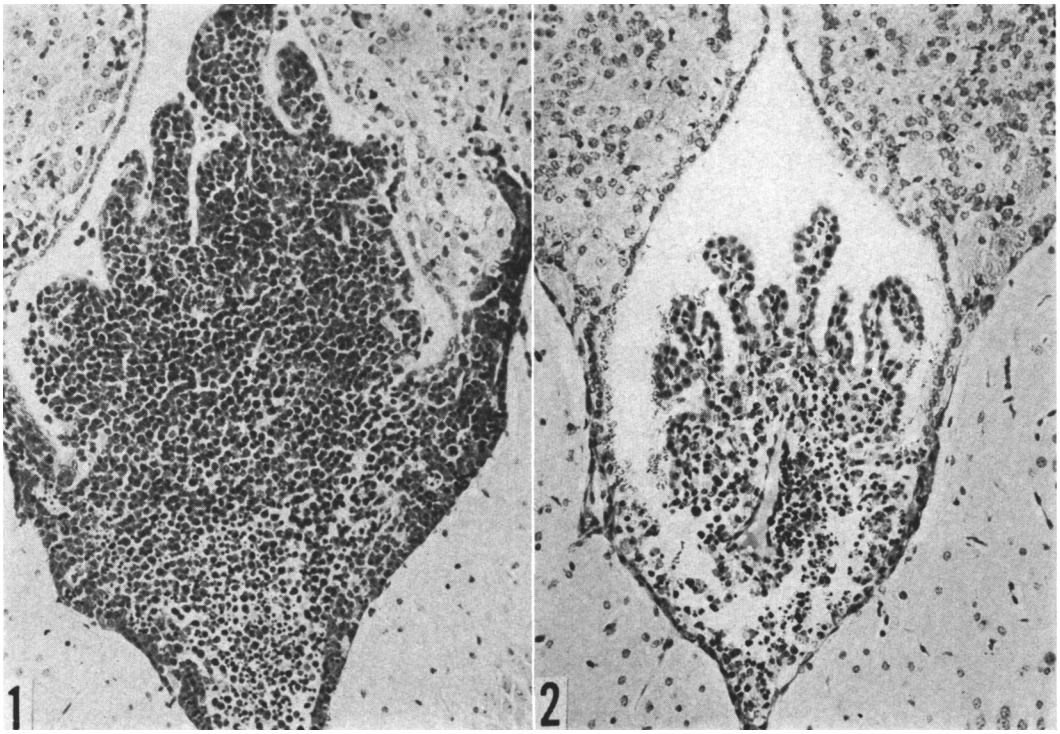

FIG. 1. Gardner lymphoma growing as solid sheet in tela choroidea in third ventricle 9 days after intracerebral inoculation with 1 x 10^6 tumor cells. (Haematoxylin and eosin stain × 130.)

FIG. 2. Tumor in tela choroidea of third ventricle 48 hr after intraperitoneal injection of 15 μ/g of *E. coli* L-asparaginase. There has been extensive tumor-cell necrosis and reabsorption of dead-cell products. Day 9 after tumor inoculation. (Haematoxylin and eosin stain × 146).

was recorded for PPLAFI and the *E. coli* enzyme (Table I). The ratio of the specific activities revealed that 0.062 mg of protein of *E. coli* enzyme showed the same L-Ase activity *in vitro* as obtained from 1 mg of PPLAFI protein.

Mean day to death. In Exp. 1 through 4, the mean day to death was recorded of animals given tumor cells intracranially, with or without subsequent L-asparaginase intraperitoneally (Table II). An increase in mean time to death of treated animals was observed to be 67% to over 367% as compared with non-treated animals in all 4 experiments in which L-asparaginase was given at the dose of 15,000 u/kg body weight or higher.

TABLE I. Specific Activity of L-Asparaginase from Guinea Pig Serum and *E. coli*.

Source of L-asparaginase	u/cc (μM NH_3 Lib/hr)	mg protein/cc	Specific activity
Guinea pig serum, partially purified Fraction I			
Exp 1	225	64	3.5
" 2	285	75	3.8
" 3	225	64	3.5
Avg, Exp 1, 2, 3	245	68	3.6
E. coli — Exp 4	1,090	19	57

S.A. ratio: $\frac{E.\ coli}{\text{PPLAFI}} = \frac{57}{3.6} = 16.$

Histopathology. Controls. Mice were examined 7-9 days after tumor implantation. Grossly, their brains were not enlarged, though the ventricles were slightly dilated and filled with white tumor. Microscopically tumor grew over the brain in the subarachnoid space as sheets, several cells in depth, and within the ventricles, particularly in the tela choroidea, as large solid-cell masses (Fig. 1). The tela choroidea always became enlarged and often occupied most of the ventricular space. Frequently, small columns of tumor cells infiltrated the paraventricular white matter for short distances, but growth of tu-

TABLE II. Effect of Guinea Pig Serum Partially Purified Serum L-Asparaginase (PPLAFI) and *E. coli* L-Asparaginase (ECLA) Injected Intraperitoneally on C3H Mice Implanted with 6C3HED Lymphosarcoma Intracerebrally.

Animals				Treatment			Sacrificed for histopathology				
No.	Body wt, avg	Source	Tumor cells	L-Ase origin, u/g	Days after tumor cells inoculated	Mean days to death	No. of animals	Days after tumor inoculated	Survivors at day of sacrifice	Histopathology and fate of tumor cells	Clinical symptoms at sacrifice
Exp 1				PPLAFI							
8	(19)	CU*	3×10^3	16	6	>26.1	6	31	6	Tumor present in brain and most viscera	Lethargy, hunched
"	"	"	"	48	"	>25.1	"	"	"	*Idem*	*Idem*
"	"	"	"	None	—	15.1	0	—	0	—	—
Exp 2				PPLAFI							
7	(17 g)	CU	1×10^6	15	3	19.4	—	—	—	—	—
"	"	"	"	45	"	14.4	—	—	—	—	—
"	"	"	"	None	—	6.0	—	—	—	—	—
Exp 3				PPLAFI							
4	(14.5)	CU	3.4×10^3	48	7	—	4	8	4	Focal necrosis of tumor in brain	Normal
"	"	"	"	"	"	—	"	9	"	Extensive necrosis of tumor in brain	"
"	"	"	"	"	"	—	2	14	2	—	"
"	"	"	"	None	—	—	4	8	4	Tumor in ventricles, meninges & tela choroidea	Lethargic
"	"	"	"	"	—	—	"	9	"	*Idem*	"
2	"	"	"	"	—	—	1	14	1	—	Head grossly enlarged
Exp 4				ECLA							
10	(27)	HVA*	1×10^6	15	6	>49.3	6	60	6	Tumor in one brain only; no tumor in 5 brains; calcified tela choroidea	Normal
"	"	"	"	45	"	>49.6	8	"	8	No tumor in brains; calcified tela choroidea	"
"	"	"	"	0	—	13.5	0	0	0	—	—
5	"	"	—	15	6	—	5	7	5	—	—
"	"	"	—	"	"	—	"	8	"	—	—
4	"	"	1×10^6	"	"	—	4	7	4	Focal necrosis of tumor in brain	—

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mor was not prominent in the solid matter of the brain at this time. The rapid growth of the tumor in the tela choroidea was one feature which did not simulate metastatic growth of lymphatic leukemia cells in the human brain.

Along the needle track were reactive glial cells, many with engulfed degenerating erythrocytes, and rarely small clumps of viable appearing tumor cells in the gray and white matter. Tumor cells were not seen in any abdominal or thoracic viscera at this time.

Treated mice. The tumor regressed within 24 hours after one intraperitoneal injection of either PPLAFI or *E. coli* L-asparaginase. There was pyknosis, necrosis and reabsorption of dead tumor cells both in the ventricles and solid matter of the brain with collapse of the tela choroidea. Only small foci of viable appearing tumor cells remained either in the tela choroidea or meninges, and these were often perivascular. By 48 hours, there was extensive tumor necrosis and reabsorption of dead-cell debris (Fig. 2). Several mice had no tumor visible in either the ventricles or meninges. Cytotoxic effects were not seen in the brain, heart, lungs, stomach, intestines, liver, kidneys, spleen, pancreas, adrenal glands, and abdominal somatic muscle of mice given *E. coli* L-asparaginase and others given PPLAFI when compared with non-treated mice (Table II).

Follow-up. Treated mice 31 days after PPLAFI. In all mice treated with PPLAFI, tumor returned by 30 days and was systemic with infiltration of nearly all abdominal viscera including kidneys, pancreas, liver, along the visceral and parietal peritoneum, often the lungs, and with diffuse infiltration of gray and white matter of the brain.

Treated mice 60 days after *E. coli* L-Ase. In 14 of 20 mice which were alive at this time and sectioned for histology, only one had a tumor and it was limited to the brain. In the other 13 mice, all had focal calcification of the tela choroidea (Fig. 3), and occasionally some inflammatory cells, but no tumor cells, were visible. Changes in other viscera consisted of focal myocardial calcification and rarely renal tubular hyaline casts.

Discussion. It is significant that PPLAFI

TABLE II (continued)

Animals			Treatment			Sacrificed for histopathology					
No.	Body wt, avg	Source	Tumor cells	L-Ase origin, u/g	Days after tumor cells inoculated	Mean days to death	No. of animals	Days after tumor inoculated	Survivors at day of sacrifice	Histopathology and fate of tumor cells	Clinical symptoms at sacrifice
4	(27)	HVA	1×10^6	15	6	—	"	8	"	Marked necrosis and regression of tumor in brain; two mice had no residual tumor	—
5	"	"	—	—	—	—	5	7	5	—	—
"	"	"	—	—	—	—	"	8	"	—	—
"	"	"	1×10^6	—	—	—	"	7	"	Extensive tumor growth in ventricles, meninges & tela choroidea	Lethargy
"	"	"	"	—	—	—	"	8	"	*Idem*	"

* CU = Cumberland View Farms; HVA = Hines Veterans Admin. Hospital, Hines, Ill.

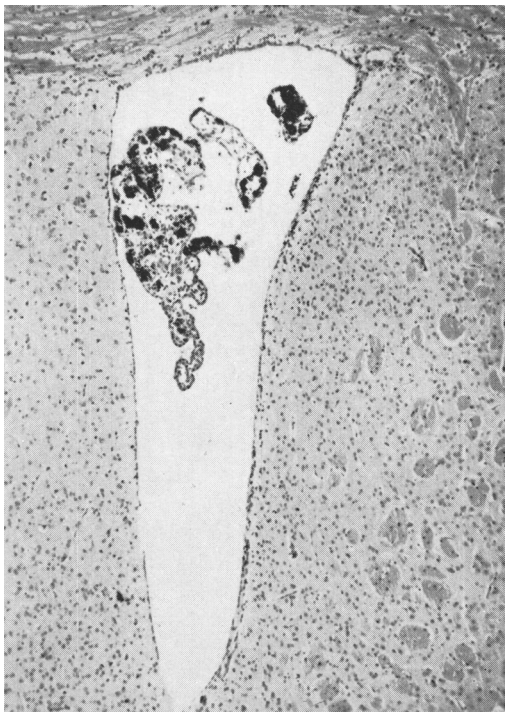

FIG. 3. Section through lateral ventricle 54 days after treatment with 45 μ/g *E. coli* L-asparaginase. No residual tumor. Dark masses in tela choroidea are areas of calcification. (Haematoxylin and eosin stain approx. × 41.)

was effective in prolonging the life of mice with intracerebral tumors and in causing necrosis of the tumor cells, since whole guinea pig serum was not effective in a previous experiment. The better effect of PPLAFI was possibly due to our ability to administer a larger dose of L-asparaginase in one treatment than was possible with whole guinea pig serum.

The fact that *E. coli* L-asparaginase was effective in doses of the same order of magnitude as the serum enzyme in terms of total L-asparaginase activity, and more effective in terms of total protein injected lends support to the hypothesis that the antitumor activity is related to L-asparaginase activity. This is not unreasonable in view of the apparent *in vitro* requirement of this line of 6C3HED tumor cells for asparagine. It is not known whether the drug penetrates the meninges or brain, but it is conceivable in view of previous evidence that it may exert its primary effect at a site removed from the tumor. However, it is not possible to draw conclusions from these data as to the relative antitumor activity of one unit of L-asparaginase of guinea pig *versus E. coli* enzyme. Precise experiments using animals of similar age and weight inoculated with tumor cells from a single pool are now under way to make this comparison.

Systemic involvement in the PPLAFI treated mice after 31 days probably occurred from tumor spreading along the central nervous system and along the dorsal roots. The distribution of the tumors differed considerably from that which occurs after subcutaneous implantation. Tumor necrosis in brain is very rapid, similar to that which occurs in subcutaneous tumors after guinea pig serum injection(5). Calcification of the tela choroidea which occurs rarely in the mouse(8) is probably secondary to the tumor cell necrosis. Animals with subcutaneous tumors did not develop calcification of the tela choroidea when examined over 60 days after *E. coli* L-asparaginase treatment in other experiments now under way in our laboratory.

Summary. Both guinea pig L-asparaginase and *E. coli* L-asparaginase, when given in sufficient doses, are effective in causing prolonged regression of intracerebral 6C3HED lymphomas actively growing in C3H mice. This suggests that further antitumor trials in other species including toxicologic experiments with larger doses appear warranted.

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