SUPPRESSION OF MURINE LEUKEMIAS BY L-ASPARAGINASE

INCIDENCE OF SENSITIVITY AMONG LEUKEMIAS OF VARIOUS TYPES: COMPARATIVE INHIBITORY ACTIVITIES OF GUINEA PIG SERUM L-ASPARAGINASE AND ESCHERICHIA COLI L-ASPARAGINASE*

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The finding that certain neoplasms have a requirement for L-asparagine, an amino acid commonly regarded as nutritionally nonessential, provides the basis of a unique form of cancer therapy.

In 1953, Kidd (1, 2) observed that certain transplanted murine leukemias were suppressed by treatment with guinea pig serum. The work of Broome (3–5) established that the active factor in guinea pig serum is L-asparaginase.[1] His main evidence was as follows: First, the chemical properties of the leukemia-inhibitory factor did not differ from those of L-asparaginase of guinea pig serum, and in various fractionation procedures the leukemia-inhibitory activity was always associated with L-asparaginase activity. Secondly, 6C3HED, the leukemia used in his studies, was found to require L-asparagine for optimal growth in culture, and cells cultured continuously in the presence of L-asparagine retained their sensitivity to suppression by guinea pig sera in vivo; on the other hand, sublines of 6C3HED that had lost their requirement for L-asparagine as a result of culture in vitro in media lacking L-asparagine were no longer suppressed by guinea pig serum in vivo. Finally, sublines of 6C3HED that had been made resistant to guinea pig serum by exposure to subeffective doses in vivo did not require L-asparagine for immediate growth in culture.

Later studies provide further evidence that the leukemia-inhibitory factor in guinea pig serum is L-asparaginase. First, the sera of animals closely related to the guinea pig (three genera of the same superfamily, *Cavioidea*) have both L-asparaginase and leukemia-inhibitory activities (6, 7); in contrast, no serum lacking L-asparaginase is effective against leukemias (1, 6–8). Secondly, L-asparaginase prepared from a bacterial source, *Escherichia coli*, suppresses 6C3HED and the DBA/2 leukemia P1534

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[1] L-asparagine amidohydrolase (EC 3·5·1·1)

(9). Subsequently, two enzymes, called EC-1 and EC-2 were separated from *E. coli*; they differ in their chemical properties and only one of them (EC-2) has leukemia-inhibitory activity (10).

Susceptibility to suppression by guinea pig serum is now known to be a common property of certain classes of leukemia in the mouse (11). Susceptibility is not confined to murine leukemias; the Murphy-Sturm lymphosarcoma of the rat (1) and two other rat tumors, Walker carcinosarcoma 256 (12) and ACMCA2 sarcoma (13), are reported to be susceptible.

This communication is concerned with a comparison between L-asparaginase from guinea pig serum and L-asparaginase from *E. coli* in the treatment of the transplanted radiation-induced leukemia EARAD1. In addition, we have extended our previous survey of murine leukemias (11) to a total of 109 leukemias that have now been tested for susceptibility to guinea pig serum.

Materials and Methods

Leukemias.—All the leukemias used in this study were induced, or arose spontaneously, in mice of our colonies. Methods of induction by X-radiation, chemical carcinogens, and leukemia viruses (Gross, Friend, Moloney, Rauscher) are described in previous publications from this laboratory (see reference 14). The leukemia used in most of the studies reported here, EARAD1, was induced by X-radiation in a (C57BL/6 × A)F_1 hybrid female of our colony in 1961 and has been maintained exclusively by passage in isogenic (C57BL/6 × A)F_1 ♀ mice. At the time of these experiments, it was in its 27th–148th transplant generations.

Guinea Pig Serum (GPS).—Hartley guinea pigs weighing more than 350 g were bled from the heart under ether anesthesia, the sera were pooled after separation and centrifugation, and the pools were stored at −70°C. The L-asparaginase activity of these pools ranged from 115–140 units/ml.

Tests of Leukemias of Various Types for their Sensitivity to GPS.—In the survey summarized in Table III leukemias were tested for sensitivity to GPS by inoculating a counted number of viable cells (usually 1×10^6) subcutaneously into 10 isogenic mice, five of which received 1 ml of GPS intravenously and 1 ml intraperitoneally within an hour of the inoculation of the leukemia cells, the other five serving as controls. Sensitivity to GPS was indicated by either complete protection or marked prolongation of survival time.

Enzyme Assays.—The L-asparaginase assays were carried out as described elsewhere (10). One unit of activity is that quantity of enzyme which releases 1 μmole of ammonia from L-asparagine per hour at the maximum rate. The clearance of GPS and *E. coli* (EC-1 and EC-2) L-asparaginases was determined by injecting the stated amount of GPS or enzyme preparation intravenously or intraperitoneally as indicated in Fig. 5. Blood was obtained by cardiac puncture from two mice for each time interval, and the pooled serum was stored frozen until assayed for L-asparaginase.

Preparation of the L-asparaginases EC-1 and EC-2 from E. coli B cells.—The preparation of EC-1 and EC-2 is described fully in a separate report dealing with the chemistry of the two enzymes (10). In summary, a cell-free extract of *E. coli* B cells is treated with $MnCl_2$ to precipitate nucleic acids and the two enzymes are partially separated by fractional precipitation with ammonium sulphate; further separation of EC-1 and EC-2 is effected by chromatography on hydroxylapatite and DEAE-cellulose columns. With the exception of the EC-1 preparation used in studying clearance rates (Fig. 5), all the experiments reported here were performed with the leukemia-inhibitory L-asparaginase EC-2.

RESULTS

Sensitivity of Leukemia EARAD1 to GPS.—Table I shows the effect of GPS administered in various doses and by various routes on the outgrowth of 1×10^6 cells of the GPS-sensitive leukemia EARAD1 inoculated subcutaneously approximately 1 hr before the administration of GPS. The subcutaneous route (distant from the site of leukemia inoculation), the intraperitoneal route, and the intravenous route of injection were almost equally effective. This pool of

TABLE I

Influence of Dosage and Route of Administration on the Inhibitory Action of Guinea Pig Serum

Leukemia: EARAD1 (C57BL/6 × A) F_1*

Groups	Treatment		No. mice without leukemia/No. mice tested	Survival time of leukemic mice
	Volume of GPS	Route		
	ml			*days*
Controls	—	—	0/8	21, 22, 24, 24, 24, 24, 24, 24
I	0.1	s.c.	0/4	25, 25, 27, 28
		i.p.	0/4	24, 25, 30, 32
		i.v.	0/4	22, 25, 27, 28
II	0.25	s.c.	0/4	27, 28, 28, 29
		i.p.	1/4	30, 32, 27
		i.v.	1/4	30, 32, 33
III	0.5	s.c.	4/4	All survived
		i.p.	4/4	
		i.v.	4/4	
IV	1.0	s.c.	3/3	All survived
		i.p.	3/3	
		i.v.	3/3	

* GPS injected approximately 1 hr after inoculation of leukemia cells.

GPS produced complete suppression of EARAD1 at a dose of 0.5 ml; a dose of 0.1 ml caused slight prolongation of survival but no absolute protection.

Table II indicates that the effectiveness of GPS is related to the number of leukemia cells inoculated. With 1 ml GPS injected intravenously at the time of inoculation of leukemia cells there was complete protection of all mice inoculated with 1×10^6 EARAD1 cells and of some mice inoculated with 3×10^6 cells. There were no survivors in groups receiving 9×10^6 or more cells. Increased survival time was observed in all mice given GPS, regardless of the number of cells in the inoculum.

Survey of a Series of Murine Leukemias for their Sensitivity to GPS.—Table III is a summary of our experience up to the present time regarding the incidence of sensitivity to GPS among leukemias of several types. The 109 leukemias listed in Table III were for the most part in very early transplant generations and include the 18 leukemias reported previously (11). Sensitivity is evidently a common characteristic of certain classes of leukemia; in particular, of leukemias induced by X-radiation (21/48) and of leukemias arising spontaneously in strains that have a low incidence of leukemia, 5/14 in the C57BL/6, A, BALB/c, I and DBA/2 strains. Of leukemias arising spontaneously or induced in strains that have a high incidence of leukemia, sensitivity to GPS is uncommon, 1/12 in the AKR and C58 strains. No instance of sensitivity was

TABLE II

Influence of the Number of Leukemia Cells Inoculated on the Inhibitory Action of Guinea Pig Serum

Leukemia: EARAD1 (C57BL/6 × A) F_1*

No. leukemia cells inoculated subcutaneously ($\times 10^6$)	No. mice without leukemia/No. mice tested		Survival time of leukemic mice, *days*	
	Controls (no GPS)	Treated with GPS	Controls (no GPS)	Treated with GPS
1	0/4	4/4	24, 24, 24, 24	All survived
3	0/5	2/5	20, 20, 20, 24, 24	34, 45, 52
9	0/4	0/4	19, 19, 19, 20	24, 28, 30, 45
12	0/4	0/4	19, 19, 19, 20	28, 28, 34, 58
24	0/4	0/4	19, 19, 19, 20	28, 28, 28, 28
48	0/3	0/3	17, 20, 20	24, 28, 30, 45

* 1 ml GPS injected intravenously approximately 1 hr after inoculation of leukemia cells.

found among 16 leukemias induced by the Gross, Friend, Moloney, and Rauscher leukemia viruses.

Effectiveness of GPS in Comparison with a Number of Commonly Used Chemotherapeutic Agents.—Three leukemias, two of them GPS-sensitive, and one GPS-resistant, were selected for the experiments shown in Table IV. With the exception of their response to GPS all three leukemias showed approximately similar sensitivities to each particular chemotherapeutic agent. In other words, the two GPS-sensitive leukemias did not differ from the GPS-resistant leukemia in their responses to this range of cytotoxic drugs. A striking feature of such comparative studies, in addition to the remarkable effectiveness of GPS in affording absolute protection, is that GPS-treated mice show no weight loss or signs of toxicity. (Survival times of mice with EARAD1 were not affected by the administration of 2.5 g/kg D-asparagine or L-asparagine daily for 10 days.)

Comparative Effectiveness of E. coli L-asparaginase and GPS, under Various Conditions of Testing, in the Treatment of Leukemia EARAD1.—

TABLE III

Susceptibility to Suppression by Guinea Pig Serum of 109 Mouse Leukemias

Strain of origin	Mode of induction									
	Spontaneous		X-Radiation		Urethan		DMBA		Virus	
	Sens.	Res.	Sens.	Res.	Sens.	Res.	Sens.	Res.	Sens.	Res.
C57BL/6	1	2	11	12	1	6				3M 3R
(C57BL/6 × A)F_1			6	8		1				
A	1	2	2	3						1M
BALB/c		2	1	2		1				3G 3M
C3H/An				1				2		1M
(BALB/c × C3H/An)F_1			1	1		1				
C58		4						1		
I	2	3								
AKR		1			1	1		4		
DBA/2	1							5		1F
(C3H/An × I)F_1						2				
C3Hf/Bi										1G
Total.....	5	14	21	27	2	12		12		16

Abbreviations: M, Moloney virus; G, Gross (Passage A) virus; F, Friend virus (transplanted solid tumor variant); R, Rauscher virus; DMBA, 9,10-dimethyl-1,2-benzanthracene; Sens., sensitive to suppression by GPS; and Res., resistant to suppression by GPS.

TABLE IV

Comparative Sensitivities of Two GPS-Sensitive Leukemias and One GPS-Resistant Leukemia to Various Chemotherapeutic Agents

	Total daily dose*	EARAD1 (radiation-induced)	RADA1‡ (radiation-induced)	ASL1‡ (spontaneous)
		Per cent increase in survival time (5 mice per group)§		
Guinea pig serum	3 ml day 0 (i.p. and s.c.) 2 ml day 1 (s.c.)	5/5 survived	−1	+93 (1 mouse) 4/5 survived
6-Mercaptopurine	30 mg/kg	+3	+12	−12
Prednisone	30 mg/kg	+5	+4	+11
Guanylhydrazone	50 mg/kg	+20	+6	+20
NSC 60339 ‖	16 mg/kg	+4	+4	+1
Methotrexate	1 mg/kg	+36	+36	+22
Azaserine	10 mg/kg	+111	+95	+81
5-Fluorouracil	13 mg/kg	+41	+19	+11
Cytoxan	200 mg/kg (one dose)	+80 (4 mice) 1/5 survived	+41	+39
Vincristine	0.2 mg/kg	+7	+3	−34
Mitomycin C	1 mg/kg	+12	+16	−21

* With the exception of GPS, cytoxan, and prednisone, all compounds were administered in 10 daily doses, starting 4 hr after subcutaneous inoculation of 10^6 leukemia cells. Prednisone was injected for 10 days twice daily subcutaneously, except for day 0 when the total daily dose was given in one intraperitoneal injection. Cytoxan was given on day 0 only. With the exceptions noted, all injections were intraperitoneal.

‡ Origins of these leukemias of the A strain are given in reference 15.

§ Based on survival times of 20 control mice treated with the diluent (saline or carboxymethylcellulose), or not injected.

‖ NSC 60339 = 2-chloro-4′,4″-bis(2-imidazolin-2-yl)terephthalanilide.

Treatment at the time of inoculation of leukemia cells: Groups of mice were inoculated subcutaneously with 1×10^6 EARAD1 cells and were treated within 1 hour with *E. coli* L-asparaginase EC-2 intraperitoneally in a dose of 50, 200, or 2000 units, or with GPS in dose of 50 or 200 units L-asparaginase. Fig. 1 shows the result of this experiment. During the first 12 days after inoculation GPS gave suppression at 200 units/mouse and partial suppression at 50 units. EC-2 was completely ineffective at 50 and 200 units; 2000 units EC-2 was less effective than 50 units GPS L-asparaginase. The survival times of these mice are given in Table V where it is seen that EC-2 gave no increase in survival time even in the highest dose of 2000 units. GPS at 200 units gave prolonged survival of three mice and permanent protection of one mouse.

Thus EC-2 administered at the time of inoculation of leukemia cells confers virtually no protection.

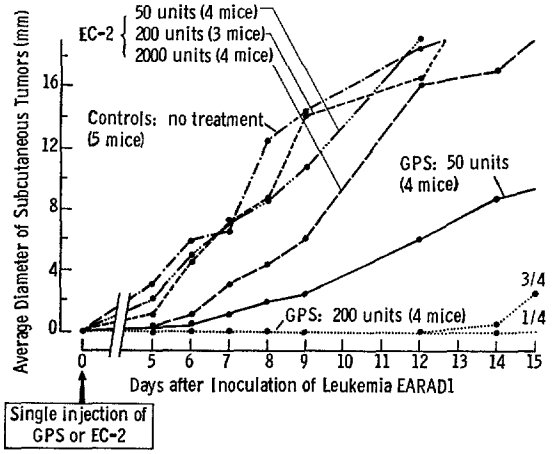

FIG. 1. Comparative effectiveness of GPS and *E. coli* EC-2 administered intraperitoneally within 1 hr after subcutaneous inoculation of leukemia EARAD1.

Treatment commencing 7 days after inoculation of leukemia cells: Fig. 2 shows the effect of a single injection of 50, 200, or 2000 units of EC-2 or of a single injection of GPS containing 50 or 200 units, administered 7 days after subcutaneous inoculation of 3.5×10^6 EARAD1 cells. At this time the subcutaneous mass is approximately 1 cm in diameter and the leukemia is generalized (demonstrated by transmitting the leukemia with cells taken from the spleens of animals inoculated subcutaneously 7 days previously). In contrast to the failure of EC-2 to protect when administered at the time of inoculation of EARAD1, EC-2 was highly effective in the treatment of these established 7-day transplants. Guinea pig serum, on the contrary, is relatively less effective when administered after the leukemia is established. Suppression by EC-2 was most marked at 2000 units, with one permanent cure in the group of four mice (Table V).

TABLE V

Comparison of Guinea Pig Serum L-asparaginase with E. coli L-asparaginase EC-2 in the Treatment of Leukemia EARAD1 Inoculated Either (a) on the Same Day, or (b) 7 Days Previously*

No. of mice	Source of L-asparaginase	Units	Day of death				
Total dose as one injection on day 0							
5	None (controls)	—	15	16	16	17	20
4	GPS	50	20	21	25	25	
4	*E. coli*	50	16	17	17	19	
4	GPS	200	25	40	47	>120	
3	*E. coli*	200	16	17	20		
4	*E. coli*	2000	17	19	19	20	
Total dose as one injection on day 7							
5	None (controls)	—	16	16	16	16	19
4	GPS	50	16	16	17	17	
4	*E. coli*	50	17	18	18	19	
4	GPS	200	19	20	21	23	
4	*E. coli*	200	19	21	22	22	
4	*E. coli*	2000	34	35	42	>120	
Ten doses on days 7–11							
4	GPS	200 (20U × 10)	20	20	21	22	
4	*E. coli*	200 (20U × 10)	20	21	21	22	

* The same preparation of *E. coli* L-asparaginase and of GPS was used for the entire experiment shown in this Table.

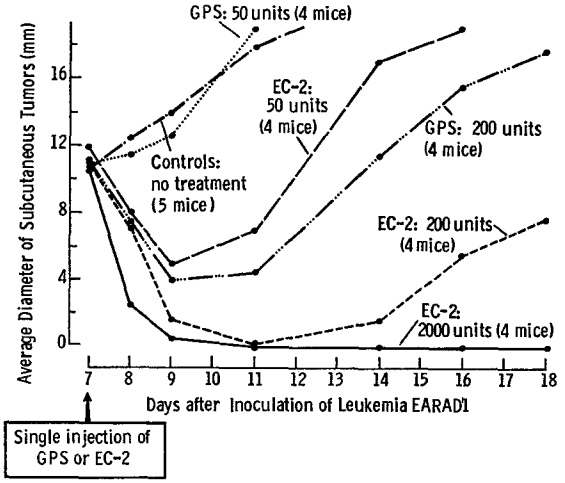

FIG. 2. Comparative effectiveness of GPS and *E. coli* EC-2 administered intraperitoneally 7 days after subcutaneous inoculation of leukemia EARAD1.

Fig. 3 shows the effect of divided doses of EC-2 or GPS administered on days 7–11 (the day of inoculation being regarded as day 0). Again EC-2, at a dose of 200 units (20 units × 10), was markedly more effective than GPS in suppressing the local tumor.

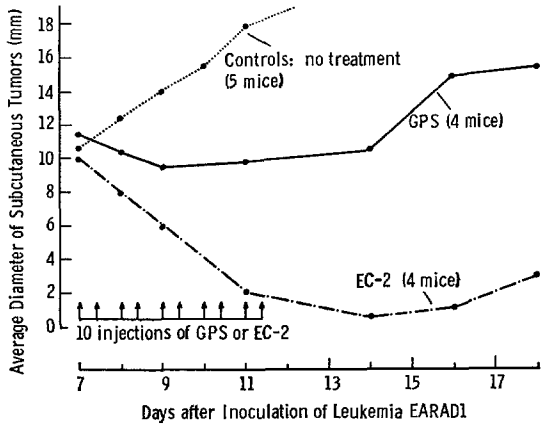

FIG. 3. Comparative effectiveness of GPS and *E. coli* EC-2 administered intraperitoneally on days 7–11 after subcutaneous inoculation of leukemia EARAD1. (20 units × 10: total 200 units).

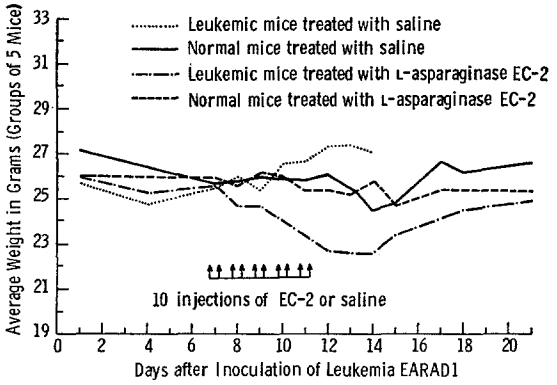

FIG. 4. Effect of 10 intraperitoneal injections of 200 units EC-2 (total 2000 units in 5 days) on the weights of normal mice and mice with 7-day transplants of leukemia EARAD1.

Treatment of nonleukemic animals with EC-2 preparations of the type used in these tests does not cause loss of weight or obvious toxicity, although leukemic animals lose weight which they recover after treatment (Fig. 4).

Permanent Cure of Mice with Established Leukemic Transplants by Treatment with EC-2 Beginning on the 7th Day after Inoculation of Leukemia Cells.—A high proportion of mice with 7-day transplants of EARAD1 can be permanently

TABLE VI

*Permanent Cure of Mice with 7-Day Transplants of EARAD1 by E. coli L-asparaginase EC-2**

Mouse No.	Total units (i.p.)	Died on day	Survivors	Notes
4108	1000	23	0/6	
4123	(100 × 10)	23		
4084		25		
4089		25		
4093		27		
4017		24		
4125	2000	—	5/6	Rechallenged 6 months later with 50,000 cells s.c.: all died of leukemia.
4133	(200 × 10)	—		
4073		—		
4131		—		
4091		—		
4085		31		
4098	4000	—	4/5	Rechallenged 6 months later with 50,000 cells s.c.: all died of leukemia.
4099	(400 × 10)	—		
4090		—		
4136		—		
4104		(37)		Sacrificed with recurrence on day 37‡
4109	None (controls given	17	0/12	
4126	buffer alone)	21		
4105		16		
4106		17		
4045		19		
4086		22		
4134		20		
4092		15		
4118		18		
4097		17		
4135		17		
4132		18		

(C57BL/6 × A)F_1 ♀ leukemia EARAD1 inoculated s.c. on day 0 into (C57BL/6 × A)F_1 ♀ mice (average weight, 20 g). Treatment started on day 7, at which time the subcutaneous mass measured 10 × 10 mm (average). 10 injections (two per day) on days 7–11. In all treated animals the s.c. mass was no longer visible or palpable on day 9.

* Specific activity: 315 units/mg protein.

‡ Transplanted and identified as resistant variant by failure to respond to GPS.

cured by treatment with 2000 or more units of EC-2. All survivors from the experiment shown in Table VI were observed for at least 6 months after treatment. As these 6-month survivors were fully susceptible to rechallenge with minimal numbers of EARAD1 cells (Table VI) it is clear that cure must be

ascribed solely to the action of L-asparaginase and not to any immune reaction accompanying therapy with L-asparaginase.

Blood Clearance of GPS L-asparaginase and E. coli L-asparaginase.—Two L-asparaginases, EC-1 and EC-2, have been isolated from *E. coli* (10). They can be partially separated by $(NH_4)_2SO_4$-fractionation and they differ in chromatographic properties and in enzymatic activity in relation to pH. Only EC-2 has leukemia-inhibitory activity.

Fig. 5 shows clearance data for GPS L-asparaginase and for EC-1 and EC-2 from *E. coli*. GPS L-asparaginase is cleared from the serum at a slow rate (half-life after equilibriation approximately 26 hr for GPS injected intravenously). By

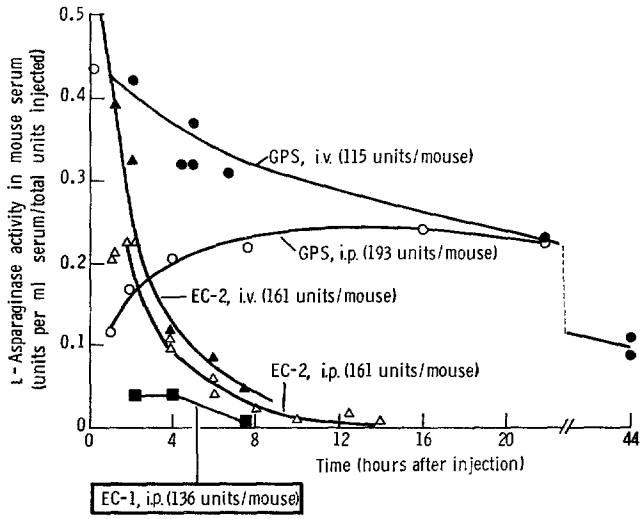

FIG. 5. Clearance of GPS L-asparaginase, EC-1 L-asparaginase, and EC-2 L-asparaginase after intraperitoneal or intravenous inoculation.

contrast, EC-2 is cleared rapidly, the rates for intraperitoneal and intravenous injection being similar except for the period of equilibration (half-life approximately 150 min). No rate of clearance from the blood can be estimated for enzyme EC-1 because apparently very little of the enzyme left the peritoneal cavity, or alternatively the half-life of the enzyme in vivo is very short (Fig. 5).

DISCUSSION

As a preliminary to discussion of the findings reported here we may consider the accumulated evidence that the inhibitory factor in GPS is L-asparaginase and that the inhibition of GPS-sensitive neoplastic cells is a consequence of a specific nutritional requirement for L-asparagine.

1. Fractionation of GPS (3, 4, 16–20) shows that fractions with inhibitory activity against GPS-sensitive leukemias invariably contain L-asparaginase.

2. Sera that contain L-asparaginase will suppress GPS-sensitive leukemias; sera that do not contain L-asparaginase do not suppress these leukemias (1, 6–8).

3. Four murine leukemias and one tumor of the rat that have been found to require L-asparagine for their growth in vitro, (6C3HED (3, 5), EARAD1 (17), L5178Y (reference 21 and unpublished observations), P1798 (unpublished observations), and Walker carcinosarcoma 256 (22)), are sensitive to suppression by GPS in vivo. Two other cell lines are known to require L-asparagine in vitro, (Jensen rat sarcoma (23) and a hepatoma of the rat (24)), but these have not been tested for sensitivity to GPS in vivo. Conversely, many murine cell lines are known to have no requirement for L-asparagine in vitro and are not sensitive to GPS in vivo. During a recent study in this laboratory 12 transplanted murine leukemias that were known to be insensitive to GPS in vivo were successfully grown in culture and none of these exhibited a requirement for L-asparagine (B. Stambuck, B. Williamson, E. A. Boyse, and L. J. Old, in preparation).

4. Cells that lose their requirement for L-asparagine in vitro are no longer suppressed by GPS in vivo and leukemia cells that have been rendered insensitive to GPS by suboptimal treatment in vivo no longer require L-asparagine for growth in vitro; this has been shown for 6C3HED (3, 5), and for EARAD1 and P1798 (unpublished observations).

5. As reported here and previously (9) L-asparaginase from *E. coli* is as active as GPS against GPS-sensitive leukemias. The great phylogenetic disparity between these two sources makes it virtually certain that the inhibitory factor in GPS is L-asparaginase and not a protein with properties closely related to L-asparaginase; it suggests furthermore that the inhibitory action derives from its deamidating activity and not from other regions of the L-asparaginase molecule.

The finding that certain leukemias and other tumors have a specific nutritional requirement for L-asparagine constitutes the only established instance of a defined biochemical difference between neoplastic cells and their normal precursors. The suppression of such leukemias by L-asparaginase thus constitutes the only specific therapy known at the present time for any form of cancer. The extraordinary effectiveness of L-asparaginase is particularly well illustrated when seen in comparison with various standard chemotherapeutic agents (Table IV). The virtually all-or-none response to L-asparaginase (depending upon the susceptibility or resistance of particular leukemias) contrasts with the less discriminating and less effective responses to treatment by cytotoxic drugs and hormones, which are exerting differential effects upon neoplastic and normal tissues. Clearly, the absence of toxicity of L-asparaginase, which makes it possible to treat with very high doses, derives from its specificity of action.

The detection of leukemia-inhibitory activity of L-asparaginases from different sources is dependent upon the circumstances under which the enzymes are assayed. GPS L-asparaginase is most effective when administered at the same time as the leukemia cells, under which condition *E. coli* L-asparaginase confers no protection. In the treatment of established tumors that have already grown to a considerable size, however, EC-2 is considerably more effective than

GPS (on the basis of equivalent numbers of units administered). A striking difference between the GPS and *E. coli* enzymes is the more rapid clearance of the latter (Fig. 5). The failure of EC-2 in the concurrent treatment of inoculated leukemia may be partly attributable to its rapid clearance, but this cannot be the only factor as it *is* effective in the treatment of *established* transplants. It is necessary to postulate that the requirement for L-asparagine is related to phases of the growth cycle of asparagine-dependent cells. Shortly after transplantation the cells may be metabolically inactive and the demand for asparagine correspondingly reduced. Later, when the cells enter a phase of growth their requirement for asparagine is presumably increased and at this time they may become susceptible to asparagine-deprivation once more. This would account for the effectiveness of GPS in the treatment of newly inoculated leukemia cells because asparagine-deprivation in this case would persist beyond the lag-phase of the inoculated cells, whereas in the case of the rapidly cleared EC-2, serum and tissue asparagine may already have returned to levels adequate for growth of the leukemia cells.

The inactivity of other L-asparaginases such as *E. coli* EC-1 (10) and yeast L-asparaginase (25) can be explained on the basis of exceedingly rapid clearance or inactivation. Other contributory factors may include low activity at physiological pH values; e.g., EC-2 has maximal activity in vitro at pH 7.4 whereas EC-1 has no more than 77% of its optimal activity at this pH (10).

The permanent cure of mice with disseminated leukemia by treatment initiated 7 days after inoculation is unique, as it is the only instance of its kind in a fully histocompatible system. The conclusion that immunological responses play no part in the recovery of these mice is emphasized by the finding that they are fully susceptible to rechallenge with minimal inocula of the same leukemia. The occurrence in EARAD1 of variants that do not require asparagine appears to be rare, or cures would not be possible. At somewhat lower levels of treatment, recurrence is the rule, but the recurrent leukemia is susceptible to retreatment, indicating regrowth of the sensitive line. As L-asparaginase is effective in retreatment of mice with recurrent leukemia or cured mice receiving a second transplant it is clear that sensitization to L-asparaginase does not occur under these conditions, or that any antibodies which may have been formed do not interfere with enzyme activity.

Of the many important questions raised by the work on L-asparaginase perhaps the most pressing is whether human tumors may be susceptible to this form of therapy. The occurrence of susceptible leukemias in mice, and of susceptible tumors of other kinds in the rat, makes it impossible to form an opinion as to which class of human tumor might respond. However, the availability of an effective enzyme from bacterial sources will make it possible to carry out adequate trials with a wide variety of tumors and leukemias in species other than small rodents. The development of a test capable of revealing asparagine-

dependence of primary tumors obviously would be of great value, but regardless of such tests final evaluation is bound to depend upon tests in vivo.

SUMMARY

A survey of 109 recently derived leukemias of the mouse revealed that sensitivity to suppression by guinea pig serum is a common property of transplanted leukemias of certain classes. The sensitive leukemias included five that arose spontaneously in mice of strains with a low incidence of leukemia and 21 that were induced by X-radiation.

Two GPS-sensitive leukemias were not more sensitive than a GPS-resistant leukemia to a range of standard chemotherapeutic agents.

The effectiveness of L-asparaginase EC-2 from *Escherichia coli* in suppression of the GPS-sensitive leukemia EARAD1 depends upon the conditions of assay. Whereas it is not inhibitory when administered as a single dose at the time of inoculation of the leukemia it is considerably *more* effective than GPS when used in the treatment of established leukemia.

Permanent cures of 7-day generalized transplants of EARAD1 can be effected by the administration of 2000 or more units of EC-2. Immunological factors apparently do not contribute to cure as treated survivors are fully susceptible to rechallenge with minimal numbers of cells from the same leukemia. Reinoculated survivors with progressively growing transplants have been successfully retreated with EC-2.

The blood clearance of EC-2 L-asparaginase injected into mice is much more rapid than that of GPS L-asparaginase. After intraperitoneal inoculation of the EC-1 L-asparaginase, which does not have leukemia-inhibitory activity, only very low levels of enzyme activity could be detected in the serum.

The effectiveness of EC-2 from *E. coli* and its availability from a virtually limitless source will make it possible to extend the study of inhibition of leukemias and other tumors by L-asparaginase to species other than small rodents.

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BIBLIOGRAPHY

1. Kidd, J. G. 1953. Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. *J. Exptl. Med.* **98:** 565.
2. Kidd, J. G. 1953. Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. II. Studies on the nature of the active serum constituent: histological mechanism of the regression; tests for effects of guinea pig serum on lymphoma cells *in vitro*: discussion. *J. Exptl. Med.* **98:** 583.
3. Broome, J. D. 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature.* **191:** 1114.

4. Broome, J. D. 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. I. Properties of the L-asparaginase of guinea pig serum in relation to those of the antilymphoma substance. *J. Exptl. Med.* **118:** 99.
5. Broome, J. D 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. II. Lymphoma 6C3HED cells cultured in a medium devoid of L-asparagine lose their susceptibility to the effects of guinea pig serum *in vivo*. *J. Expt. Med.* **118:** 121.
6. Old, L. J., E. A. Boyse, H. A. Campbell, and G. M Daria. 1963. Leukaemia inhibiting properties and L-asparaginase activity of sera from certain South American rodents. *Nature.* **198:** 801.
7. Holmquist, N. D. 1963. Effect of normal sera of several related rodents on 6C3HED lymphoma *in vivo*. *Proc. Soc. Exptl. Biol. Med.* **113:** 444.
8. Herbut, P. A., and W. H. Kraemer. 1958. The effects of animal serums on lymphosarcoma 6C3HED in C3H mice. *Am. J. Pathol.* **34:** 767.
9. Mashburn, L. T., and J. C. Wriston, Jr. 1964. Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. *Arch. Biochem. Biophys.* **105:** 450.
10. Campbell, H. A., L. T. Mashburn, E. A. Boyse, and L. J. Old. Two L-asparaginases from *E. coli B*: Their separation, purification and antitumor activity, *Biochem istry,* submitted for publication.
11. Boyse, E. A., L. J. Old, and E. Stockert. 1963. Inhibitory effect of guinea pig serum on a number of new leukaemias in mice. *Nature.* **198:** 800.
12. Kwak, K. S., E. Jameson, R. M. Ryan, and H. M. Kurtz. 1961. The effect of varying implant cell numbers on the inhibitory activity of guinea pig serum on Walker carcinosarcoma 256 in the rat. *Cancer Res.* **21:** 44.
13. Jameson, E., H. Ainis, and R. M. Ryan. 1956. Action of guinea pig serum and human gamma globulin on the growth of a rat tumor. *Science.* **124:** 980.
14. Old, L. J., E. A. Boyse, and E. Stockert. 1965. The G (Gross) leukemia antigen. *Cancer Res.* **25:** 813.
15. Old, L. J., E. A. Boyse, and E. Stockert. 1963. Antigenic properties of experimental leukemias. I. Serological studies *in vitro* with spontaneous and radiation induced leukemias. *J. Nat. Cancer Inst.* **31:** 977.
16. Mashburn, L. T., and J. C. Wriston, Jr. 1963. Tumor inhibitory effect of L-asparaginase. *Biochem. Biophys. Res. Commun.* **12:** 50.
17. Campbell, H. A., L. J. Old, and E. A. Boyse. 1964. Leukemia inhibitory and L-asparaginase activities of guinea pig serum. *Proc. Am. Assoc. Cancer Res.* **5:** 10.
18. Suld, H. M., and P. A. Herbut. 1965. Guinea pig serum and liver asparaginases; purification and antitumor activity. *J. Biol. Chem.* **240:** 2234.
19. Yellin, T. O., and J. C. Wriston, Jr. 1966. Antagonism of purified asparaginase from guinea pig serum toward lymphoma. *Science.* **151:** 998.
20. Hiramoto, R., C. Tate, and M. Hamlin. 1966. Guinea pig serum inhibitory factor and asparaginase activity. *Proc. Soc. Exptl. Biol. Med.* **121:** 597.
21. Haley, E. E., G. A. Fischer, and A. D. Welch. 1961. The requirements for L-asparagine of mouse leukemia cells L5178Y in culture, *Cancer Res.* **21:** 532.
22. Neuman, R. A., and T. A. McCoy. 1956. Requirement of Walker carcinosarcoma 256 *in vitro* for asparagine and glutamine. *Science.* **124:** 24.

23. McCoy, T. A., M. Maxwell, and P. F. Kruse, Jr. 1959. The amino acid requirements of the Jensen sarcoma *in vitro*. *Cancer Res.* **19:** 591.
24. McCoy, T. A., and M. Maxwell. 1959. Some nutritional aspects of a 3′-methyl-4 dimethylaminoazobenzene-induced hepatoma *in vitro*. *J. Nat. Cancer Inst.* **23:** 385.
25. Broome, J. D. 1965. Antilymphoma activity of L-asparaginase *in vivo*: clearance rates of enzyme preparations from guinea pig serum and yeast in relation to their effect on tumor growth. *J. Nat. Cancer Inst.* **35:** 967.