LEVELS OF ENZYMES IN LEUKAEMIC MICE TREATED WITH *AEROMONAS* L-ASPARAGINASE

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ABSTRACT

L-asparaginase isolated in our laboratory from *Aeromonas* has been found to be antileukaemic. In the present study changes in the levels of serum enzymes in leukaemic mice and under treatment with *Aeromonas* L-asparaginase has been compared. A significant increase in the levels of serum lactate dehydrogenase with tumour growth and a decrease during therapy was observed. A significant decrease in alanine transaminase activity during tumour growth and an increase during treatment was noticed. Increased levels of aspartate transaminase and alkaline phosphatase was observed during enzyme therapy. Total acid phosphatase was found to be increased during tumour growth and decreased considerably during treatment.

KEY WORDS : Enzymes, *Aeromonas*, L-Asparaginase, Leukaemia

INTRODUCTION

An antileukaemic enzyme L-asparaginase, from *Aeromonas* was isolated in our laboratory (1,2) and found to be nearly as effective as *E. coli* L-asparaginase (Leunase). But our enzyme is superior in the sense that it has only one form and the total enzyme is antileukaemic. Where as *E. coli* L-asparaginase has two forms (EC1 & EC2), of which only one form is active making the enzyme more attractive pharmaceutically L-asparaginase is effective in the treatment of acute lymphatic leukaemia. This enzyme hydrolyses L-asparagine to aspartic acid and ammonia. Sensitive tumour cells are unable to synthesise L-asparagine. So protein synthesis is inhibited in sensitive cells in the absence of L-asparagine or in the presence of L-asparaginase. Normal cells are able to synthesise L-asparaginase with the help of asparagine synthetase and do not require exogenous supply of L-asparagine(4). L-asparaginase depletes the

exogenous asparagine making the cancer cells deficient in Asparagine for the growth and multiplication.

Metabolism of carbohydrate, (4-7) proteins (4,8-9) and glycoproteins (10-11) in leukaemia and under treatment with *E. coli* L-asparaginase have been studied. Many workers have reported hypoinsulinemic hypoglycemia during the treatment of L-asparaginase(4). With *Aeromonas* L-asparaginase there are only few studies on its antileukaemic effects and some alterations in the metabolism of glycoproteins (12) and lipids (13) in leukaemic cells.

There are no detailed studies on the alteration of enzyme activities in leukaemic cells which could possibly be used as markers while under treatment with *Aeromonas* L-asparaginase. Since L-asparaginase treatment affected liver functions and were manifested by elevated liver

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enzymes(4), the effect of other antileukaemic drugs including leunase was studied on transaminases, acid and alkaline phosphatases and lactate dehydrogenase. So we also investigate the effect of *Aeromonas* L-asparaginase on the enzymes transaminases, acid and alkaline phosphatases and lactate dehydrogenase.

MATERIALS AND METHODS

Inbred Balb/c mice of 7 weeks age, weighing 14-17 g were used for the study. The mice were fed on Gold mohur rat feed, (Hindustan Lever Ltd, Bangalore) and water ad-libitum. Ehrlich ascites tumour cell line, kindly provided by Amala Cancer Research Institute, Trissur was used for the study. The tumour cell line was maintained by serial intraperitoneal (IP) transplantation in mice. Full grown tumour cells were aspirated from the mouse peritoneum, washed thrice with 0.9% saline, and suspended in saline. About 1×10^5 cells were injected intraperitoneally into a new healthy mouse. All operations were done aseptically. Solid tumour growth was obtained from the ascites cells by injecting 5×10^4 cells subcutaneously under an area of shaven skin on the flank.

Growing of cells, preparation of crude cell free extract and purification of enzyme were as described by Benny(2). The enzyme preparation obtained after ammonium sulfate precipitation (80% saturation), gel permeation hydroxyapatite chromatography and lyophilization was dissolved in 0.9% saline. *E. coli* asparaginase, (Leunase) purchased from Biochem India Ltd. was used to compare the antineoplastic activity of *Aeromonas* L-asparaginase. The lyophilised enzyme preparation was dissolved in 0.9% saline.

Four groups of six mice weighing 14 -17 g were used for the study. Group I were normal mice with laboratory feed and Group II (control group) untreated Ehrlich Ascites tumour implanted mice. About 1×10^5 tumour cells were injected into the peritoneal cavity. These mice were sacrificed on the 8th and 15th day subsequent to tumour implantation. Group III consisted of tumour implanted mice treated with Leunase 24 hrs. after tumour implantation. Leunase was administered by the same route in three intermittent doses of 25 units at an interval of 48 hrs. The mice were sacrificed on the 8th and 15th day of tumour implantation. In group IV tumour implanted mice treated with *Aeromonas* L-asparaginase. Injections (as in the case of Leunase) were used. They were also sacrificed on the 8th and 15th day of tumour implantation. The diameter of the subcutaneous mass was measured (average of two measurements at right angles) on the 7th and 12th day. The maximum survival period of mice in each group was also checked for a period of 60 days. After overnight fasting the mice in each group were killed by cutting the jugular vein. Levels of total lactate dehydrogenase, aspartate and alanine transaminases, acid and alkaline phosphatases were assayed in serum by the methods described by King (14), Clermont and Chalmers (15), King and Jagantheesan (16), and Boyer (17) et. al. respectively.

RESULTS

The data on reduction of solid tumour by L-asparaginase from *Aeromonas* and Leunase are presented in tables 1,2 and 3. Activities of the enzymes viz. lactate dehydrogenase, transaminases, acid and alkaline phosphatases in tumour and L-asparaginase treated and untreated tumour bearing mice are summarised in tables 4

Table 1. Comparison of antineoplastic activity of Leunase and *Aeromonas* L-asparaginase

Dose of L-asparaginase (IU)	Mean change in tumor size (mm)*				
	Control	<i>Aeromonas</i> L-asparaginase	Effect %	Leunase	Effect %
0 Units	8.6				
5 Units		+3.1	+ 36	+3.0	+ 34.88
10 Units		-2.8	- 32	-2.9	- 33.72
25 Units		-6.5	- 75.58	-6.9	- 80.23
50 Units		-8.1	- 94.18	-8.4	- 97.67
3 intermittent doses of 10 Units each		-8.4	- 97.67	-8.5	- 98.83

* Diameter of the tumour mass 6 mice in each group

Table 2. Survival rate of mice treated with L-asparaginase from *Aeromonas* on Ehrlich ascites tumour bearing mice

Dose of L-asparaginase (IU)	No. of injections	'Cures'/mice treated*	Percentage of survivors**	Day of death	Day of sacrifice of tumour free mice
Control (not treated)	1	0/6	0	14,16,18, 19,22,22	-
10	1	2/6	100	34,39,43, 56	60 (2 mice)
25	1	4/6	100	28,54	60 (4 mice)
50	1	3/6	100	45,51,55	60 (3 mice)
100	1	1/6	100	38,47,48, 53,58	60 (1 mice)
3 intermittent 60 (6 mice) doses of 25 units each	3	6/6	100		60 (6 mice)

* Mice that survived up to the 60th day of experiment.

** Survivors at the time of death of control. L-asparaginase was administered intraperitoneally in a single dose, 24 hrs after the tumour implantation.

Table 3. Leunase from E.coli and Aeromonas L-asparaginase on the growth of Ehrlich ascites tumour in mice

Dose of L-asparaginase (IU)	Leunase		Aeromonas L-asparaginase	
	Average survival time (days)	Increase in life span (%)	Average survival time (days)	Increase in life span (%)
Control (not treated)	18.5	100	18.5	100
10 units	57.4	310.2	50.7	274
25 units	61.5	332.4	65.2	352.4
50 units	65.7	355.1	60.7	328
100 units	62.8	339	51.5	278.4
3 intermittent doses of 25 units each	88.6	479	84.8	458

Table 4. Serum transaminases and Lactate dehydrogenase levels in tumour bearing and L-asparaginase treated mice

Groups	LDH activity	ALT activity	AST activity
Normal	16.18 ± 1.17	26.66 ± 1.81	23.33 ± 1.26
8 days post tumour implant	37.53 ± 1.84 ^a	21.66 ± 2.07 ^a	24.54 ± 1.59
Treated with Leunase	28.12 ± 1.95 ^a	38.33 ± 2.36 ^a	29.66 ± 1.73 ^a
Treated with Aeromonas L-asparaginase	31.36 ± 2.17 ^a	36.57 ± 2.74 ^a	29.53 ± 1.64 ^a
15 days post tumour implant	42.72 ± 2.86 ^a	27.49 ± 2.05	31.48 ± 2.21 ^a
Treated with leunase	34.95 ± 2.74 ^a	39.87 ± 1.87 ^a	28.31 ± 1.94
Treated with Aeromonas asparaginase	38.45 ± 3.11	31.47 ± 1.39 ^a	30.47 ± 1.47

Values are expressed as IU/ 100 ml serum; Average of 6 values in each group ± SEM; Groups 2 & 5 have been compared with Group 1; Groups 3 & 4 have been compared with Group 2; Groups 6 & 7 have been compared with Group 5; a- p less than 0.01; b- p between 0.01 and 0.05.

and 5 respectively. When 5 units of *Aeromonas* L-asparaginase was injected subcutaneously, there was reduction in the growth rate of tumour, compared to controls. 10 units of enzyme could bring down the tumour size (3 folds). 50 units of L-asparaginase injected into the subcutaneous tumour mass caused almost complete regression (94%) of the tumour mass. Enzyme injections were administered 7 days after tumour implantation and the effect was measured on the 12th day. Better results (97%) were obtained when 3 intermittent doses of 10 units were injected at an interval of 24 hrs. Such decrease (98%) in tumour mass was also obtained with *E. coli* L-asparaginase at the same dose levels. Average reduction in tumour mass was more with increase in the dose of L-asparaginase (from 10 U-50 U). Complete disappearance of the subcutaneous tumour mass without any tissue necrosis was obtained with both *Aeromonas* and *E. coli* L-asparaginase, injected in 3 doses of 10 units each. It can be seen that all the animals which received 75 units of *Aeromonas* L-asparaginase intraperitoneally in 3 intermittent doses, survived till the end of the experiment. Administration of *Aeromonas* enzyme as a single dose increased the average survival period of Ehrlich ascites tumour bearing mice (at all doses) when compared with untreated controls (Table 3). Even with the smallest dose of 10 IU, survival period was 57.4 days. Further increase in dose up to 50 IU resulted in only slight increase in survival time. However, when the dose was further increased to 100 IU, there was no benefit. On the other hand the average survival period was less (62.8 days) than that with 50 IU (65.7 days). The average survival period could be increased by injecting the same amount of enzyme (25 IU) in 3 intermittent doses (88.6 days) than with as a single dose (61.5 days).

Alteration of liver functions in subjects treated with L-asparaginase is documented (4). There was a significant rise in the serum level of lactate dehydrogenase with tumour growth. There was only a partial but significant reversion in the level of this enzyme during therapy with both L-asparaginases (table 4). However Leunase was more effective than *Aeromonas* L-asparaginase. Few days after the cessation of therapy, this reversion became less significant (not shown in the table). There was significant decrease in alanine transaminase level in the serum with tumour growth and very significant increase was observed immediately after L-asparaginase treatment. This increase in alanine transaminase activity remained during later stage in animals treated with both *E. coli* and *Aeromonas* L-asparaginase. Treated groups showed a significant rise (68%) in serum alanine transaminase activity. Serum aspartate transaminase showed an increase which was not significant during the early phase of tumour growth and remained as such during the later phase. There was significant ($p < 0.01$) rise (21%) in the serum level of this enzyme immediately after L-asparaginase administration in both the cases. Total acid phosphatase in serum was found to be increased significantly during the early phase of tumour growth. In the later phase of tumour growth it was higher but less than that after 8 days of tumour implant. Tartrate labile acid phosphatase, increased considerably during early stage. During the early stage of treatment with both the enzymes there was decrease but still the values were significantly higher than the controls ($p < 0.01$). In later phase, also there was an decrease in both the cases. In fact, the change in total acid phosphatase was almost fully due to changes of tartrate labile fractions. Administration of L-asparaginase (25 U x 3 doses) caused a reduction

in the total as well as tartrate labile serum acid phosphatase levels.

Serum alkaline phosphatase showed a significant increase (66%) with tumour growth (Table.5). There was a significant rise in the serum immediately after L-asparaginase administration and at a later phase, no significant difference was found between the *Aeromonas* treated and non-treated groups. But in *E. coli* asparaginase treated groups, the enzyme level was found to be decreased slightly but not significantly. The study has shown that tartrate labile acid phosphatase level was found to be increased with tumour and decreased in early phase but not in later phase with *Aeromonas* asparaginase treatment.

DISCUSSION

Both *E. coli* and *Aeromonas* L-asparaginases were having curative effects at a dose level of 25 units. The average life span was found to be increased 274%, 352%, 328%, 458% and 278% when mice were injected with 10 U, 25 U, 50 U, 75 U (25 U x 3) and 100 U of enzyme preparation respectively. All the animals survived for an average of 84.6 days when 75 units were injected in three intermittent doses of 25 IU each. The successful regimen of intermittent doses of L-asparaginase has already been reported (18). Gaffar stated that the reduction in the survival period when large doses (100 U) were injected could be due to the toxicity of the enzyme preparation (19). Small doses of L-asparaginase from *Aeromonas* were curative whereas high dose did not show the same trend which may be due to toxicity of the enzyme as mentioned above. The average life span was slightly higher but not significant in mice treated with *Aeromons* L-

asparaginase when compared with *E coli* L-asparaginase when 25 IU of enzyme preparation was administered. Better results were obtained with *E. coli* and *Aeromonas* L-asparaginases, when these were injected in intermittent doses. Similar trend was also noticed with L-asparaginase isolated from *Vibrio* (18). The results clearly establish the antineoplastic property of L-asparaginase, on Ehrlich ascites tumour, purified from estuarine *Aeromonas* and is equally comparable with that of *E. coli* L-asparaginase (Leunase). The antineoplastic activity of this enzyme is due to the depletion of exogenous L-asparaginase and by inhibiting the synthesis of proteins in tumour cells where asparagine synthetase is lacking. The present study shows that L-asparaginase from estuarine *Aeromonas* is also effective in inhibiting the growth of Ehrlich ascites tumour in mice. A significant increase (16.18 U to 37.53 U) in the levels of serum lactate dehydrogenase with tumour growth and partial reversion (37.53 U to 31.36 U) during treatment with both L-asparaginases observed in this study is an index of tumour burden and in response to therapy as suggested by Vasanthi et. al. (20). However the reversion was not very significant and couldn't be used for monitoring therapy. A significant decrease in alanine transaminase activity in serum was observed during tumour growth and an increase in the activity during therapy might be used as an index of diagnosis and for monitoring therapy. Serum aspartate transaminase level was found to be increased but not significant in tumour and remained elevated significantly during treatment with both the enzymes contrary to the suggestions that elevated serum transaminases in tumour growth manifest alterations in liver function caused by nutritional deficiency that reverted to normal after therapy. Total acid phosphatase was found to be increased

Table 5. Serum phosphatase levels in tumour bearing and L-asparaginase treated mice

Groups	Total acid phosphatase	Tartrate labile acid phosphatase	Alkaline phosphatase
Normal	2.71 $\pm$ 0.165	1.23 $\pm$ 0.083	4.17 $\pm$ 0.203
8 days post tumour implant	6.78 $\pm$ 0.481 ^a	5.71 $\pm$ 0.236 ^a	6.31 $\pm$ 0.424 ^a
Treated with Leunase	5.36 $\pm$ 0.253 ^a	3.57 $\pm$ 0.127 ^a	8.52 $\pm$ 0.672 ^a
Treated with Aeromonas L-asparaginase	5.13 $\pm$ 0.237 ^a	3.24 $\pm$ 0.143 ^a	7.84 $\pm$ 0.347 ^a
15 days post tumour implant	4.29 $\pm$ 0.186 ^a	2.53 $\pm$ 0.151 ^a	6.66 $\pm$ 0.295 ^a
Treated with leunase	5.28 $\pm$ 0.214 ^a	2.29 $\pm$ 0.177 ^b	6.23 $\pm$ 0.368
Treated with Aeromonas asparaginase	5.05 $\pm$ 0.226 ^a	2.47 $\pm$ 0.128	7.14 $\pm$ 0.386

Values are expressed as King Armstrong units IU/ 100 ml serum.

Average of 6 values in each group $\pm$ SEM.

Group 2 & 5 have been compared with Group 1.

Group 3 & 4 have been compared with Group 2.

Group 6 & 7 have been compared with Group 5.

a- p less than 0.01.

b- p between 0.01 and 0.05.

during tumour burden and the regression was observed in treated groups during the early stage of the treatment. In our study tartarate labile acid phosphatase level could be used as a tumour maker for diagnosis of cancer and for monitoring therapy in the early phase only. Serum alkaline phosphatase was found to be increased in cancer and remained elevated during treatment with L-asparaginase contrary to the observations of Bates et al.²¹

The L-asparaginase isolated from estuarine *Aeromonas* was found to be antineoplastic and the total enzyme is active against tumours. Other factors that might affect the antitumour property of L-asparaginase include pH optima, K_m value and serum half life (K_m) of the enzyme in the host. Good activity of *Aeromonas* L-asparaginase at the physiological pH, stability at elevated temperature (40°C), and high substrate affinity of this enzyme (low K_m value ie 1.75×10^{-5} M) (2,22) etc. would also explain antitumour property of *Aeromonas* L-asparaginase.

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