ANTICANCER ACTIVITY OF L-ASPARAGINASE: A REVIEW

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GENERAL INTRODUCTION

Cancer has become one of the most disturbing killer diseases and a national problem for developed as well as developing countries. The main reasons for not divising an effective way of checking this neoplastic disease are its obscure origin, late detection and development of resistance by the host to the chemotherapeutic agents which are non-specific, immunosuppressive and some are even teratogenic. Among all the anticancer drugs from alkylating agents, antimetabolites, hormones, plant products and antibiotics, L-asparaginase has been proved to be very effective against several leukemias, lymphomas and other neoplastic diseases in animals as well as in man. Because of this novel and very useful biological property, this enzyme demands a special attention among hundreds of enzymes so far studied from various biological sources. The success in the use of L-asparaginase in cancer treatment is based on the hydrolysis of extracellular L-asparagine, an obligatory requirement for the growth of many leukemias and lymphomas.

The establishment of the biochemical difference—absolute requirement of exogenous L-asparagine for the growth of some types of leukemias and lymphomas and the therapy of such tumours by L-asparaginase, is rather interesting. First, Kidd [1] observed the inhibitory effect of guinea pig serum on the growth of mouse leukemias. Later, Broome [2] noticed similar inhibitory effect in tissue culture experiments and further proved that the active principle in Kidd's observation was the enzyme L-asparaginase. However, the starting point for Broome's conclusion was the observation of Clementi [3] who as early as in 1922 showed that guinea pig serum alone

among many mammalian sera, is rich in L-asparaginase. Finally, Mashburn and Wriston [4] demonstrated that the same enzyme from *Escherichia coli* can exert inhibitory effect on the growth of mouse leukemias. This discovery led to large-scale production of the enzyme from microbial source and also to a study of its experimental and clinical effect on different types of tumours.

At present L-asparaginase is produced commercially under the trade name 'Crasnitin' [5]. However, an endotoxin as well as L-glutaminase activity have been found associated with the commercial preparation. Because of the high cost in the large-scale preparation of this enzyme from *E. coli* and also because of the production of antibodies due to continuous usage from one source, various other microbial sources have been studied for obtaining immunologically noncross-reactive L-asparaginase possessing antitumour activity [6, 7, 8].

ASSAY METHOD

L-Asparaginase (L-asparagine amidohydrolase, E.C. 3·5·1·1) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. Therefore, the enzyme activity can be measured by estimating the products formed either ammonia or aspartic acid or by the disappearance of the substrate asparagine. Though several assay methods are available, ammonia estimation by nesslerization is inexpensive, reliable and highly reproducible.

DISTRIBUTION

L-Asparaginase is widely distributed in microbial, plant and animal sources. Extracellular production of this enzyme was found in many microorganisms [9] and is also detected in the periplasmic region in *E. coli* [10], and in *Pseudomonas aeruginosa* [11].

REGULATION OF L-ASPARAGINASE

Studies on regulation of L-asparaginase in microorganisms are very important in obtaining the enzyme in good yield for detailed studies on physicochemical and biochemical properties, pharmacodynamics, toxicology, experimental and clinical effects. For inducing the synthesis of enzyme protein, the effect of different sources of carbon [12, 13, 14], nitrogen [15, 16, 13, 17] and metal ions [13] have been studied in detail in several microorganisms. Studies on regulation of L-asparaginase synthesis have been

done in *P. boreopolis* 526 [18], *Erwinia* [19] and in *Azotobacter vinelandii* [20]. Factors like aeration, temperature and pH are manipulated in the growth medium for the optimal production of the enzyme. A study of the kinetics of fermentation and continuous process of asparaginase production has been carried out in detail by Liu and Zajic [23] in *E. aeroideae*. Simple methods of releasing the enzyme from *E. coli* [10] and *A. vinelandii* by using *n*-butanol [24] have been demonstrated.

Biochemical Studies

It is interesting to note that most of the biochemical studies have been carried out with L-asparaginase isolated from *E. coli*. A probable reason could be that the enzyme was isolated for the first time from *E. coli* among

TABLE I

Comparative account of some of the properties of L-asparaginases from various sources

Source	Molecular weight	Km value	pH maximum	Antitumour activity	Reference
Guinea pig serum	138,000	$7{\cdot}2 \times 10^{-5}$	7·5–8·5	Yes	(25)
E. coli	125,000	$1{\cdot}25 \times 10^{-5}$	8 (broad (maximum)	Yes	(26)
Serratia marcescens	150,000	1×10^{-4}	6·9 (broad (maximum)	Yes	(27)
Erwinia carotovora	128,000	..	..	Yes	(28)
Fusarium tricinetum	160,000	..	7·5-8·5	Yes	(29)
Bacillus coagulans	85,000	$4{\cdot}7 \times 10^{-3}$	8·5-9·5	No	(30)
Mycobacterium tuberculosis	..	$2{\cdot}0 \times 10^{-3}$	9·6	Yes	(31)
Azotobacter vinelandii	84,000	$1{\cdot}1 \times 10^{-4}$	8·6	Yes	(20)

microorganisms and was thus available in plenty. All the well-known classical enzyme separation techniques of biochemistry like salt and organic solvent fractionation, molecular sieving, ion exchange chromatography and also affinity chromatography have been successfully employed in the preparation of L-asparaginase from a variety of microbial sources. Table I gives a general account of the properties of L-asparaginase purified from various sources.

Studies on the amino acid composition of asparaginase from *E. coli* by Whelan and Wriston [26] and Arens *et al.* [32] reveal that the following five amino acids account for 50 percent of those present in the enzyme molecule: aspartic acid, threonine, alanine, valine and glycine.

Different values for isoelectric point of the *E. coli* enzyme have been reported [33, 26, 34]. The single band obtained by conducting electrophoresis on cellulose acetate paper [35] was later found when subjected to same electrophoresis for 15 hrs, to separate into four enzymatically active equispaced bands with different isoelectric points. This has been taken as indicative of the presence of four components in the enzyme [35]. But the number of subunits in the enzyme is not yet clearly demonstrated (6 subunits) [26] though majority of the evidences points to the presence of 4 subunits per molecule of asparaginase in *E. coli* [36, 32]. Progress in sequencing the enzyme has been surprisingly slow. By using Edman procedure, Arens *et al.* [32] have established the sequence of the first 14 amino acids from the N-terminal end for the *E. coli* enzyme.

Very interesting chemical and immunochemical studies have been carried out on L-asparaginase modified by chemical substitutions. A variety of reagents like glyoxal, H_2O_2-dioxane, acetyl imidazole and acetic anhydride [37] have been used to modify the amino residues of the enzyme. Such modified enzymes showed changes in some of the properties like hemeagglutination, precipitation patterns, etc., compared with the native asparaginase. Such structure-activity relation studies have been recorded by several others [38, 39, 40].

Antimicrobial and antiviral activity of L-asparaginase

Chang *et al.* [41] have observed a chemosterilant effect of asparaginase on house flies when administered either orally or parentarally. A striking inhibitory effect on the multiplication of vaccinia and myxoma pox virus in *in vitro* has been observed by Maral and Werner [42]. The enzyme was

effective when present in the medium. However, a direct effect of the enzyme on the virion was not found. Such *in vitro* viricidal action of asparaginase against herpes simplex virus types 1 and 2, and vaccinia virus was found to be increased by cytosine arabinoside and 1-amino-4-acetyl piperizine, etc. [43, 44]. Synergism has been implicated in this effect.

Asparagine metabolism in Eukaryotic cells

The only known metabolic pathways of asparagine in animal tissues are its conversion to aspartate by deamidation and to α-ketosuccinamic acid by transamination as also its direct incorporation into proteins [45]. A possibility with regard to the role of asparagine in sensitive tumour systems is that asparagine might furnish its amido group for nucleic acid biosynthesis, just as glutamine is known to do so in normal cells. The biological system in which this has so far been shown to occur is the wheat germ [46]. It is not clear whether asparagine plays an important role in some novel metabolic pathway in cancer cells. But from studies on the nutritional requirement of Walker carcinoma, Neuman and McCoy [47] have shown that glutamine and asparagine are necessary for growth in *in vitro*. The out growth of asparagine independent tumour cell variants from mixed culture of asparagine dependent Jensen sarcoma, when deprived of the amino acid, has been demonstrated by McCoy *et al.* [48].

Pharmacological aspects of L-asparaginase

Most of the asparaginase sensitive neoplasms like that in Mouse [2, 49, 4], rat [1], dog [50, 51] and in man [52, 53] have been studied with asparaginase from *E. coli*.

When the enzyme is administered intravenously, the activity could be detected in plasma. Intramuscular and intraperitoneal injection resulted in rapid absorption into the blood. When the enzyme was given orally, no enzyme activity was detected in plasma. As long as the therapy is continued, the levels of the enzyme are detected depending upon dosage. When administration is stopped, the enzyme activity gradually falls to undetectable levels. Several variations in this behaviour of various asparaginases are noticed. These in turn depend on species and age of the host. But the fall of enzyme levels in plasma during a prolonged treatment is attributed to the development of antibodies against the enzyme resulting in inactivation. Experimental studies on the partition of asparaginase between blood and lymph in rats and sheep have shown that most of the enzyme is

rapidly cleared from blood, while very small amounts are found in lymph [54]. The advantage with *E. coli* enzyme is that it can easily cross the capillary endothelium and enter the intercellular spaces. But, the enzyme from *Erwinia* possesses the tendency to aggregate spontaneously and therefore cannot be found in places other than circulatory system [54].

The half-life ($t_{\frac{1}{2}}$) of asparaginase in plasma was found to be 23 hrs, 4 hrs and 90 min in dogs, rabbits and rats, respectively [55]. In mice, infected or injected with lactate dehydrogenase elevating (LDH) virus, the clearance of asparaginase is impaired resulting in long $t_{\frac{1}{2}}$ and the animals show long term, often permanent remissions following a single dose of the enzyme [56, 57]. However, the virus itself is not responsible for any effect observed. It could be that the virus has synergistic effect with the enzyme.

The fate of the enzyme after its clearance from plasma is not yet clearly understood. It is not detected in urine when administered intravenously [58]. It has been suggested that the enzyme may be phagocytosed by the reticulo endothelial system [59].

Experimental effects of L-asparaginase

In order to have a better understanding of the effects of L-asparaginase with regard to dose, dosage schedule, effect on various stages of susceptible tumours, combination chemotherapy, $t_{\frac{1}{2}}$ of the enzyme, survival time of the host, immunosuppression, etc., studies have been carried out on experimental tumours in *in vitro* as well as in *in vivo* conditions.

The reversal of the effect of asparaginase on tumours by supplying L-asparagine through drinking water to the mice [60] clearly indicates that asparagine depletion is responsible for tumour regression.

Under *in vivo* conditions, Burchenal *et al.* [61] have observed an increase in survival period with low dose of the enzyme (10–40 IU/kg) in mice with leukemia. However, a higher dose (1000 IU/kg) has been found to give better results in few cases of tumours [62]. Studies on the effect of dose on survival time and cure rate in mice with leukemia EARAD 1 have shown that a maximum dose of 10,000 IU/kg or 5 daily doses of 2000 IU/kg gives a high cure rate. No further effect was noticed by increasing dosage [63]. The minimum daily dosage to increase survival time was found to be 200 IU/kg. Doses higher than 500,000 IU/kg were found to be toxic. An interesting observation regarding the duration of treatment was that, irrespective of the daily dose, the inhibitory effect was increasing with duration

of treatment. Experiments on therapeutic effectiveness of different schedules of treatment in mice have shown that, the cure rate obtained by injections 1200 IU/kg once a week for 4 weeks was found to be less effective than 12 injections of 400 IU/kg given 3 times a week for the same period. Administration of 24 × 200 IU/kg (6 times a week), however, did not show any cure. These points clearly demonstrate a relationship of tumour inhibition to dosage schedule. Asparaginase was found to be ineffective on leukemias when administered as a single dose at the time of inoculation of the tumour to mice but found to be very effective against the established leukemias [64].

Development of resistance by the host for the enzyme therapy is a serious drawback for continuing the treatment. But asparaginase resistant tumours are found to be responsive to other antitumour agents like actinomycin D, cytosine arabinoside, malphalan, natulan, etc. This has favoured the combination therapy using the enzyme in combination with other antitumour agents. Such studies have shown positive effects when these agents are used after the treatment with enzyme.

L-Glutaminase activity of the enzyme seems to play a role in regression of certain tumours. It has been shown that HeLa cells, being not sensitive to asparaginase from guinea pig (having no glutaminase activity), are found to be inhibited by the *E. coli* enzyme (65). Similarly, Riley *et al.* [66] have reported from *Acinetobacter* a glutaminase-asparaginase enzyme which inhibited both sensitive tumours and also tumours other than normal tumours like leukemias and lymphomas. Like most of the antitumour drugs, this enzyme was also found to be immunosuppressive. This property has been demonstrated under *in vitro* and *in vivo* conditions [67, 68].

Toxicity

Asparaginase, like other antineoplastic agents, is not totally free from side effects. Some of these effects have been attributed to the association of endotoxin and also to the glutaminase activity of the enzyme. Toxicity studies in experimental animals indicate LD_{50} for mouse and rat above 200,000 IU/kg and for cats and dogs to be 50,000 IU/kg [69]. The enzyme caused decreased motility, anorexia, weakness, vomiting, loss in body weight, etc. Subchronic toxic doses (rats and dogs, 200, 800 or 3000 IU/kg once a week) showed no considerable effect on the general behaviour, physiology and histology of the animals but antigenicity, pyro-

genicity and teratogenic effects were noticed in rabbits [70]. Similar kind of symptoms were noticed in human beings as side effects during treatment [71]. One of the serious side effects is on coagulation. It has been noticed in acute leukemic cases that the levels of fibrinogen and plasminogen are decreased during asparaginase therapy. This is attributed to an indirect effect or by some mechanisms induced by asparaginase (71). The toxicity of asparaginase has been reviewed by Haskell and Canellos [72], Canellos *et al.* [73] and Oettgen *et al.* [74].

Clinical use

The demonstration of regression of lymphosarcoma in dog by asparaginase [75] and the treatment of human patient by Dolowy *et al.* [76] have opened the way for clinical use of this enzyme. Making use of an *in vitro* test of asparagine dependency, adopted from the ^{14}C-labelled valine [77], Hill and coworkers [53] have reported remission in the case of acute lymphoblastic leukemia (ALL). Oettgen *et al.* [54] have observed similar results in the treatment of lymphosarcoma, ALL and acute myeloblastic leukemia. Prompted by these impressive results, asparaginase has been employed in the treatment of a large number of neoplastic diseases in human beings. Several important studies have been made to overcome the difficulties experienced during the clinical trials. The quick nature of its action and the presence of several toxic side effects have indicated that this enzyme could be considered as a potent agent to induce remission and to be followed by conventional maintenance treatment.

Resistance problems

Because of the development of resistant cells with high levels of L-asparagine synthetase during the treatment [72], it has been suggested that the sensitive tumours respond due to an enzymatic deletion in the genome [78, 79]. A correlation in the levels of L-asparagine synthetase in normal and malignant tissues has been shown by Morton and Nicholas [79]. From the studies of Becker and Broome [80], Adamson and Fabro [70] and Asteldi *et al.* [81], it can be concluded that there exists a spectrum of asparaginase sensitive cells in the tumours from which the resistant cells finally develop into new tumours. However, the advantage with asparaginase is that, it can induce complete remissions in ALL even at a very late stage of the disease when resistance to most other chemotherapeutic agents has developed.

The possibility of the development of antibodies to inactivate this enzyme during treatment, has been demonstrated in several instances [82, 83]. It is at this period that an immunologically non-cross reactive asparaginase will be of great use in the continuation of the enzyme therapy. Maclennon [84] has shown that the antibodies raised against *E. coli* enzyme not to cross-react with *Erwinia* asparaginase.

Use of structural analogs

Many studies on other ways of achieving the depletion of L-asparagine in checking tumour growth have been carried out. Lowering the amount of L-asparagine intake through food materials is found to have no significant effect on minimising the L-asparagine levels in plasma [85]. Several analogs of L-asparagine have been tried to lower the synthesis of L-asparagine through feedback control. Chou *et al.* [86] have described two such analogs; 5-Diazo-4-oxo-L-norvalline and 5-chloro-4-oxo-4-norvalline as effective inhibitors of L-asparagine synthesis in whole cells and in cell-free extracts.

Combination Chemotherapy

Multiple drug therapy in the case of neoplastic diseases has been proved to be superior to that of single drug [87, 88]. When asparaginase is used in combination with prednisone, vincristine, rubidomycin and methotrexate, the toxicity is not found to be additive to the toxicity of the above antineoplastic agents. The absence of cross-resistance between the enzyme and other-antitumour drugs is found to be of another great advantage for these studies. The effectiveness of the enzyme in combination with cytosine arabinoside and 6-azauridine when tested against murine leukemia in mice, a synergistic effect with the former two was observed [89]. Similarly the antitumour activity of asparaginase when given along with bis-β-chloroethyl nitrosourea [90] or with L-glutamine analogs like azaserine or azotomycin [91] against leukemias was found to be enhanced.

Maintenance of effective levels of asparaginase in plasma during the treatment plays a key role in the therapy against tumour systems. But asparaginase being a protein raises other difficulties. Some of the problems like immunogenicity, hypersensitivity, rapid rate of clearance, etc., can be overcome by administering the enzyme in a manner which prevents antibody formation. Chang [92] has described a method of enclosing asparaginase in semipermeable microcapsule into which asparagine can

easily diffuse and be hydrolysed. These microcapsules, when injected intraperitoneally, inhibited the growth of lymphosarcoma in mice. Allison *et al.* [93] have achieved the same by attaching the enzyme covalently to a nylon tube which can be conveniently introduced into the circulation.

Mode of action of L-asparaginase

The antitumour activity of the enzyme is found to be interesting because most of the tumours are extravascular and the enzyme has to penetrate from blood into the microenvironment of the tumour cells to exert the desired effect. When the enzyme is administered into the vascular system it catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia and thereby prevents the supply of the amino acid to the tissues [94]. As a consequence of this action, all the tissues in the system, more particularly L-asparagine dependent tumours are starved. Similarly, this enzyme also prevents the free diffusion of L-asparagine from the surrounding tissues to the cancerous cells. Because of the high pressures in the blood stream, the enzyme molecules can pass into the intercellular spaces from the fine capillaries and catalyse the hydrolysis of L-asparagine [55]. As a result of the hydrolysis of L-asparagine, accumulation of large amounts of L-aspartic acid [94, 95] and ammonia [53] have been reported. These in turn are found to have no role in tumour regression. The levels of L-asparagine in tumour tissue is reported to be depressed but not completely eliminated during the treatment [96, 94]. With the termination of the therapy, L-asparagine level slowly rises to the normal level while that of L-aspartic acid and ammonia decreases in the plasma. The fate of L-glutamine in blood plasma is very similar to that of L-asparagine when the enzyme possesses L-glutaminase activity. The supposed intercellular action of the enzyme is given in Fig. 1.

The cytomorphological effects of asparaginase in sensitive cells have been observed under the light and electron microscopes [97]. The enzyme is known to exert changes in histological structure in solid tumour tissue as well as in the single cells of mouse leukemias after 48 hrs of the administration of the enzyme. The results of these studies have shown most marked changes in ribosomes, mitochondria and in the formation of coarse ergastoplasm.

The primary cellular function which is affected by this enzyme is protein synthesis, followed by the synthesis of DNA and RNA [77, 98, 99]. As a result of treatment with the enzyme, few quantitative changes in other

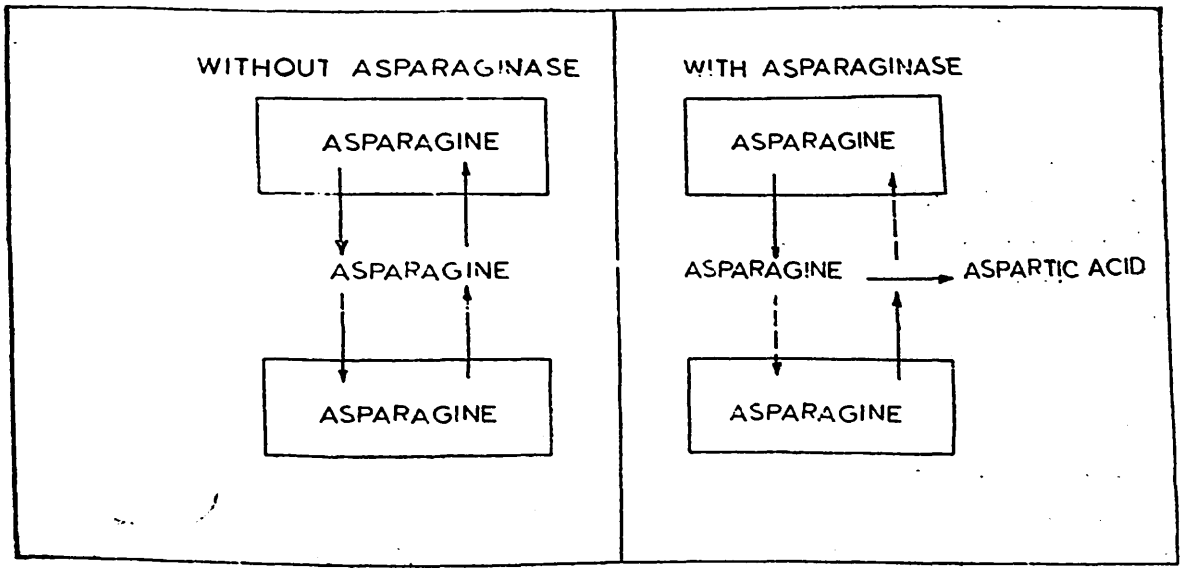

FIG. 1. Diagram of the supposed mechanism of action of asparaginase in the intercellular space (Pütter 1970).

cellular enzymes have been reported. For example, an increase in ribonuclease activity in lymphosarcoma [100, 101] and a decrease in the levels of RNA polymerase in regenerating liver [102] have been demonstrated.

Other ways of action of the enzyme in tumour regression is not totally ruled out. It has been suggested that by stimulating RNase activity [103] or by exerting limitations on the availability of the amino acid from a novel synthetic pathway in cancer cells, the enzyme may be acting as antileukemic agent. Mashburn and Wriston [100] and Mashburn and Landin [101] have recorded following asparaginase administration, a rise in RNase levels in the tumour tissue. Inhibition of glycoprotein and protein synthesis required for membrane structure in leukemic cells is considered to be another mechanism of action [104]. Glycine is important in the synthesis of purines. Decreased levels of glycine observed after the administration of enzyme might be playing a critical role in the tumour regression [105]. Inhibition of growth of HeLa cells by asparaginase at a dosage exceeding a certain limit has indicated a possibility of the enzyme partially crossing the cell membrane and exerting intracellular activity [106].

Therefore, a look at proposed mechanism of action of asparaginase indicate that, perhaps, depletion of L-asparagine is the primary mode of action which is associated and/or followed by many other alterations in biochemical events which may in fact be important secondary effects.

ROLE OF OTHER ENZYMES AS CHEMOTHERAPEUTIC AGENTS

Since the discovery of antineoplastic activity of L-asparaginase, attention has been paid for exploiting the restriction of supply of other amino acids in the chemotherapy of malignant disease. A brief account on some important enzymes studied for antitumour activity is given below. Using L-phenylalanine ammonialyase (specifically deaminates L-phenylalanine to cinnamic acid and L-tyrosine to coumaric acid), Abell *et al.*]107] observed the inhibition of growth of human leukemic and murine leukemia 5178Y lymphoblasts in *in vitro*. Similarly, L-methioninase as a depleter of L-methionine has been shown to inhibit the growth of malignant cells in tissue culture and also of an experimental tumour [108]. An important role for the enzyme L-serine dehydratase (catalyses the degradation of L-serine into pyruvate and ammonia) has been proposed in the treatment of L-serine dependent tumours [109] but clinical studies have not yet been reported. An excellent review covering the details of many enzymes as possible therapeutic agents in cancer chemotherapy is written by Cooney and Rosenbluth [110].

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