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Immobilized L-asparaginase-L-glutaminase from *Acinetobacter glutaminasificans* in microspheres: some properties in vivo and in an extracorporeal system

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Summary

The L-asparaginase-L-glutaminase used was prepared from *Acinetobacter glutaminasificans* and gave one band on gel-electrophoresis. This enzyme, with an almost 1 : 1 ratio between L-asparaginase and L-glutaminase activities, was immobilized in small, ~ 1 μm acrylamide microspheres. The immobilized enzyme was more stable than the soluble enzyme. When enzyme-containing microspheres were injected intraperitoneally in rats, the serum levels of L-asparagine and L-glutamine were rapidly reduced to low levels within 1 day. The values were not normalized until day 5. When the corresponding soluble enzyme was given, no effect on the serum levels of these amino acids was observed. Installation of small L-asparaginase-L-glutaminase containing microspheres into the outer compartment of a commercial hollow fiber dialyzer (hemofilter) gave an efficient extracorporeal reactor for decomposing circulating L-asparagine and L-glutamine. The clearance depends on the flow rate and the load of enzyme in the particles. Microsphere entrapped L-asparaginase-L-glutaminase in extracorporeal shunts may be of interest in the treatment of acute lymphoblastic leukemia (ALL).

Introduction

L-Asparaginase has been used extensively during the past 10–15 years for the treatment of acute lymphoblastic leukemia (ALL) (Lloyd and Bono, 1981; Broome, 1981; Ertel et al., 1979; Uren, 1981). The rationale, for using L-asparaginase was based on the assumption that the circulating L-asparagine is essential for the leukemic cells which appear to lack the ability to synthesize L-asparagine. However, it soon became obvious that the use of L-asparaginase is associated with several problems, especially immunological reactions such as hypersensitivity and antibody formation (Killander et al., 1976). Another problem which limited the use of L-asparaginase was the rebound phenomenon as a result of the rapid induction of liver L-asparagine synthetase (Haskell and Canellos, 1969). To overcome these problems, the enzyme was immobilized in solid drug carriers (e.g. microspheres and liposomes) giving increased stability against denaturation and reduced immunogenicity (Edman and Sjöholm, 1979; Neerunjun and Gregoriadis, 1976; Sjöholm and Edman, 1984). However, immunological reactions are best avoided by using L-asparaginase extracorporeally. Several extracorporeal systems have been developed and tested on both animals and man but they all suffer from a rapid rebound of L-asparagine (Edman et al., 1983; Jackson et al., 1979). Thus, already 24 h after treatment, the L-asparagine level is normalized. It has been reported that L-glutaminase used together with L-asparaginase

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in an extracorporeal system reduces the rebound phenomenon and that the level of L-asparagine was low even after 24 h (Giordano et al., 1981). In that report, the L-asparaginase was immobilized on the outer surface of the hollow fibers while L-glutaminase in soluble form was added to the outer compartment of the hollow fiber reactor. In the present paper we describe the purification of an enzyme from *Acinetobacter glutaminasificans* having almost equal affinity for L-asparagine and L-glutamine. This means that all effects seen may be related to a single enzyme. The purpose of this paper was to investigate the possibilities of using microparticles of polyacrylamide as carriers for L-asparaginase-L-glutaminase in an extracorporeal hollow fiber dialyzer. Furthermore, the in vivo effects of L-asparaginase-L-glutaminase immobilized in microparticles were documented.

Materials and Methods

Materials

Acrylamide and N,N′-methylenebisacrylamide (Bis) were bought from Eastman Kodak Co. Aspartate aminotransferase, glutamate dehydrogenase, malic dehydrogenase, α-ketoglutaric acid, nicotinamide adenine dinucleotide (NADH, reduced form), nicotinamide adenine dinucleotide phosphate ($NADP^+$, oxidized form), Nesslers reagent (ammonia colour reagent) were purchased from Sigma Chemicals Co. L-Asparagine, L-glutamine, L-aspartic acid and L-glutamic acid were obtained from Merck Co. Other chemicals were of commercial grade.

Animals. Normal male Sprague–Dawley rats weighing 250–300 g were obtained from Alab AB Stockholm, Sweden. The animals had unrestricted access to food and water.

Methods

Enzyme. L-Asparaginase-L-glutaminase (AGA) was obtained and purified from *Acinetobacter glutaminasificans* (ATCC 27197) following the method outlined by Roberts et al. (1972). The purity of the enzyme was checked by polyacrylamide gel electrophoresis. The specific activity of the enzyme was approximately 100–120 units/mg protein. The rate of hydrolysis of L-asparagine was 90% of that obtained with L-glutamine. The enzyme was stored as a lyophilized powder.

Enzyme assay. L-Asparaginase and L-glutaminase activities were measured by determining the amount of ammonia produced upon hydrolysis of L-asparagine and L-glutamine at +37°C as described by Roberts et al. (1972). The ammonia was determined by Nesslers reagent. Enzyme and substrate blanks were included in all assays. A standard curve was prepared with ammonium sulphate. Enzyme activity is expressed as international units (the amount of enzyme producing 1 μmol of ammonia per min at +37°C).

Preparation of enzyme-containing microparticles. Microparticles of polyacrylamide were prepared as described earlier (Edman and Sjöholm, 1979) with a slight modification. AGA (5000 IU in 50 mg) was dissolved in 2.5 ml of 0.1 M phosphate buffer (pH 7.5). The AGA solution was mixed with an equal volume of a phosphate buffer containing acrylamide (475 mg), N,N′-methylenebisacrylamide (25 mg), L-glutamine (19 mg) and EDTA (1×10^{-3} M). Ammoniumperoxo-disulphate was added to the solution before emulsification in 300 ml of chloroform–toluene (1 : 4) containing 0.09 g Pluronics F68. The polymerization was induced by addition of 500 μl N,N,N′,N′-tetramethylethylenediamine. The microparticles were washed several times with physiological saline to free them from the organic phase and non-immobilized AGA. This procedure gave a higher yield (10–12%) of immobilized enzyme than the earlier method. The particles produced (T–C = 10–5) are characterized according to the nomenclature suggested by Hjertén (1962). T describes the total amount of monomers (g/100 ml of solvent) and C the amount of N,N′-methylenebisacrylamide expressed as the percentage (w/w) of the total amount of monomers.

Determination of L-glutamine and L-glutamic acid. L-Glutamine and L-glutamic acid concentrations in solution and serum were measured by the coupled enzymatic assay described by Warrel et al. (1980). L-Glutamine is transformed by L-glutaminase to L-glutamic acid which is the substrate for L-glutamate dehydrogenase producing

α-ketoglutarate. In the latter reaction, $NADP^+$ is reduced to NADPH which can be followed spectrophotometrically.

Determination of L-asparagine and L-aspartic acid. L-Asparagine and L-aspartic acid in solution and serum were assayed by the NADH coupled method of Cooney et al. (1970).

Extracorporeal unit/reactor. AGA microparticles (2000 IU) were installed on the outer surface of the hollow fibers of a commercial hemofilter FH 202 from Gambro AB Lund Sweden as earlier described by Edman et al. (1983). The hemofilter consists of 6500 fibers with an effective membrane area of 1.2 m^2. The internal fiber diameter is 215 μm.

In vitro experiments

A phosphate buffer (5 liters) pH 7.4 containing 50 μM L-asparagine and 500 μM L-glutamine was circulated through the hemofilter units at different flow rates, 50–300 ml/min. Samples were withdrawn for assay of amino acids content before passage through the hemofilter unit and after 5, 15, 25, 45, 60, 90, 120, 150 and 200 min. The clearance values and the rate constants (k_{el}) were calculated from the concentration–time curve as previously outlined by Edman et al. (1983)

The stability of free and immobilized enzyme in phosphate buffer 0.05 M pH 7.4 were investigated at +4°C during 10 weeks. The test samples contained 0.1% sodium azid as a preservative.

In vivo experiments

Four groups of rats were used. Free and immobilized AGA, 1000 IU/kg was given to two groups of rats intraperitoneally. The two control groups were given empty microparticles or physiological saline. Blood samples were taken from the rats by heart puncture at 0, 1, 3, 5, 7 and 10 days.

Results

In vitro experiments

Stability

Stability tests were carried out at +4°C for free enzyme solution and particle suspension. The results are shown in Fig. 1. The immobilized enzyme in microparticles was more stable after 10 weeks storage than the free enzyme.

Hemofilter dialyzer

The dialyzer units with 2000 IU AGA were recirculated with 5 liters of 50 μM L-asparagine and 500 μM L-glutamine solution at flow rates of 50–300 ml/min at room temperature. Samples were withdrawn from the arterial end of the dialyzer. The rate constant (k_{el}) was determined from the slope and the clearance value from the expression:

$$\text{Clearance} = k_{el} \times V_d$$

Fig. 2 shows, that the clearance of L-asparagine and L-glutamine is dependent on the flow through the dialyzer. There was no significant difference in clearance of L-glutamine or L-asparagine. The values are almost the same and increase similarly when the flow rates are increased. At flow rates from 50 up to 200 ml/min, the clearance increases linearly from approximately 30–40 to 100 ml/min. At higher flow rates, 300 ml/min, the clearance of L-asparagine and L-glutamine deviates from the linear increase and seems to approach a plateau. Particles with a lower specific enzyme content but the equivalent amount of enzyme (2000 IU per

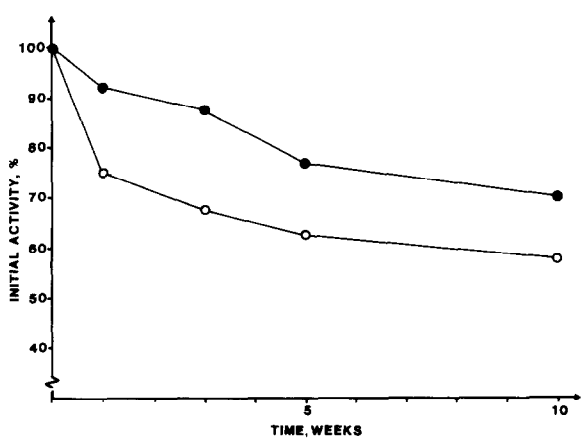

Fig. 1. Stability of soluble (○) and immobilized AGA (●) stored at 4°C. The initial enzyme activity was 27 IU/ml and 18 IU/ml, respectively, for the soluble and the immobilized enzyme.

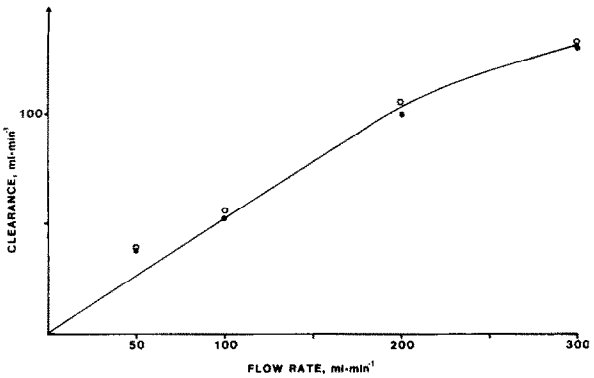

Fig. 2. Plot of clearance versus flow rate through a hollow fiber dialyzer containing 2000 IU of AGA. Five liters of buffer containing 50 μM L-asparagine and 500 μM L-glutamine were circulated at different flow rates through the dialyzer. The clearance of L-asparagine is shown by filled circles and L-glutamine with open circles.

dialyzer) give increased clearances (data not shown).

In vivo experiments

The results from the in vivo studies are shown in Figs. 3 and 4. Intraperitoneal injection of physiological saline or empty microparticles or soluble

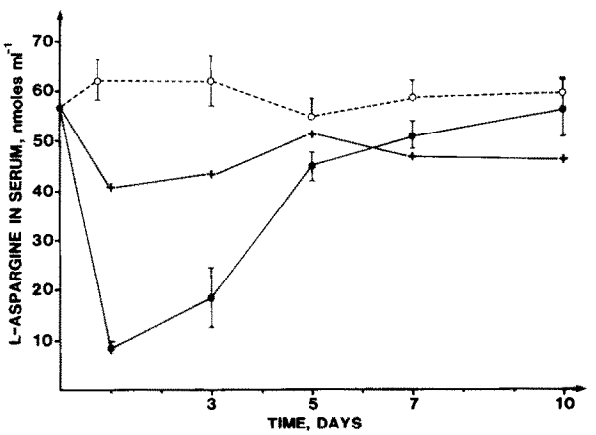

Fig. 3. Serum L-asparagine levels after intraperitoneal injection of AGA. The enzyme was given in immobilized (●) or soluble form (○). Blood samples were taken by heart puncture at times indicated and analysed as described in Materials and Methods. Experimental points represent mean ± S.E. from 4–5 rats. The normal L-asparagine level (+) was obtained from rats given physiological saline intraperitoneally. The points represent the mean from 3 rats.

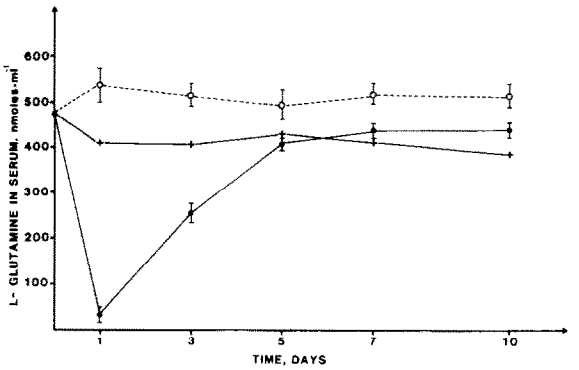

Fig. 4. Serum L-glutamine levels after intraperitoneal injection of AGA. The enzyme was given in immobilized (●) or soluble form (○). Blood samples were taken by heart puncture and analysed as described in Materials and Methods. Experimental points represent mean ± S.E. from 4–5 rats. The normal L-glutamine level (+) was obtained from rats given physiological saline intraperitoneally. The points represent the mean from 3 animals.

AGA into rats did not affect the serum concentration of L-asparagine or L-glutamine. Only normal values were seen during the time period studied. Immobilized AGA (1000 units/kg) on the other hand, given by the same route, produced an immediate drop in the L-asparagine and L-glutamine concentration from a normal value of 50 nmol/ml, respectively, 500 nmol/mm to < 10 nmol/ml and < 50 nmol/ml within 1 day. The serum levels of these amino acids were normalized after 5 days. One rat out of 5 receiving entrapped AGA did not respond to the treatment (data not shown). No effect was observed on the L-glutamine level on the day following dosage. A small effect was, however, seen on the serum level of L-asparagine, which was reduced to ~ 50% of the normal level at day 1. At day 3 the level was normalized.

Discussion

Immobilization of labile enzymes in or onto solid matrices increases the stability leading to an improved biological half-life for the enzyme, (Chang, 1972; Edman and Sjöholm, 1979; Ekman and Sjöholm, 1978). Besides increasing the stability of the enzyme, the enzymatic characteristics

are preserved. Only small alterations in K_m and V_{max} are seen (Ekman and Sjöholm, 1978).

The in vivo studies showed that soluble AGA given intraperitoneally to rats had no effect on the amino acids monitored whereas enzyme immobilized in microspheres gave a significant reduction of L-asparagine and L-glutamine levels for up to 5 days. The reason why soluble enzyme is not effective in vivo may be its instability in biological liquids or that the enzyme is removed rapidly from the circulation. Presumably both factors may contribute. However, one rat out of 5 receiving particle-entrapped AGA did not respond so markedly. It is conceivable that certain rats have a divergent intermediary metabolism with an increased L-asparagine or L-glutamine synthesis. This is supported by an observed increased rebound of L-asparagine which is seen after the initial reduction (50% of normal level) at day 1 (data not shown). This is important since it means that there may be non-responder individuals to this type of therapy. These results clearly indicate that AGA has the ability to depress the serum levels of L-asparagine and L-glutamine. Installation of AGA-microparticles in the outer compartment of a hemofilter unit created an enzyme reactor with good in vitro properties; high flow rates and clearance. The clearance is dependent on the flow and the enzyme content in the hemofilter as previously shown by Edman et al. (1983). However, the clearance values obtained with this system are somewhat lower than those obtained earlier using L-asparaginase in microparticles in a similar hemofilter. The reasons for this discrepancy may be that the particles used in this study had a higher specific enzymatic activity than previously. Thus, approximately 5 times fewer particles are needed to load each extracorporeal unit with 2000 IU of enzyme. This may result in smaller contact area between the enzyme and the hollow fiber membranes with a reduced clearance as a result.

The influence of the enzyme content per particle on the performance of the dialyzer is demonstrated by the fact that particles with a lower specific enzymatic activity gave an increased clearance. Since a greater proportion of these particles are deposited in the outer compartment and consequently the majority of the pores in the hollow fiber membranes will contain particles, a larger surface and a more efficient use of the entrapped enzyme will result.

Whether this system with AGA is better than the reported L-asparaginase system (Edman et al., 1983) in depressing the L-asparagine level has yet to be established in vivo.

The main advantage with the system consisting of a hollow fiber dialyzer and immobilized enzyme in microspheres, is that a patient may be treated with a heterologous enzyme without induction of immunological reactions: since, firstly, the cut-off of the membrane used is smaller than the molecular weight of the enzyme and, secondly, the leakage of enzyme from polyacrylamide particles is insignificant (Ekman and Sjöholm, 1978). These properties taken together will guarantee that the enzyme is not exposed to the immuno-competent cells in the blood during the treatment.

In addition to the treatment of acute lymphoblastic leukemia, there are several other applications in medicine for microparticles entrapped in extracorporeal systems, e.g. enzymatic removal of bilirubin from blood using bilirubin oxidase (Lavin et al., 1985) and the elimination of low-molecular substances from serum (Callegaro and Denti, 1983).

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