Table 2. Effect of histamine on promethazine-induced changes in transmembrane migration ratio of sperms (expressed as % of control motility) (n = 6).

Concn of promethazine (mM)	Promethazine alone (Mean ± s.d.)	Promethazine + histamine (2·5 mM) (Mean ± s.d.)	Promethazine + histamine (5·0 mM) (Mean ± s.d.)
1·0	91·8 ± 2·71	91·5 ± 2·17	97·2 ± 2·14
2·5	53·2 ± 3·37	*67·0 ± 6·78	*80·0 ± 7·62
5·0	38·0 ± 2·45	*58·0 ± 4·69	*62·3 ± 3·15
7·5	22·2 ± 4·60	*39·8 ± 3·90	*52·2 ± 3·66
10·0	15·2 ± 3·47	*24·8 ± 3·60	*38·8 ± 4·22

* Compared with promethazine alone $P < 0.01$.

Discussion

Several studies have shown that drugs with local anaesthetic properties inhibit sperm motility including procaine, propranolol (Hong et al 1981a), chlorpromazine (Hong et al 1982), and tetrahydrocannabinol (Hong et al 1981c). It is probable that the effects of chlorpheniramine and promethazine demonstrated in this study reflect their known local anaesthetic actions (Bowman & Rand 1981). The antagonism of these effects by histamine is difficult to explain, however, as histamine alone produced no significant effect, and is to be the subject of further studies. Cimetidine appears to be free of local anaesthetic properties, as it did not significantly influence sperm motility. This is consistent with its lack of local anaesthetic activity on the frog isolated sciatic nerve (Brimblecombe et al 1975).

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Potential use of an asparaginase-dextran conjugate in acute lymphoblastic leukaemia

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The relatively rapid elimination of *Erwinia carotovora* asparaginase from the circulation hampers its use in the treatment of acute lymphoblastic leukaemias. Repeated doses are required to achieve a therapeutic response and these are inconvenient to the patient. In addition, asparaginase has proved to be a potent antigen in man and repeated injections increase the risk of a hypersensitivity reaction occurring in response to the enzyme. The circulatory half-life of asparaginase has been increased by chemical modification (Blazek & Benbough (1981) and by conjugation to soluble polymers (Bendich et al 1982; Poznansky et al 1982). Benbough et al (1979) and Foster & Wileman (1979) have shown that soluble dextran-asparaginase conjugates can be prepared without extensive loss of enzyme activity, and that these conjugates have prolonged circulatory half-lives and show markedly reduced antigen reactivity (Elliott et al 1981; Wileman et al 1981). Moreover, dextran is used regularly as a plasma expander and its low toxicity is well established. This relatively simple modification procedure may have overcome the major limitations to the use of asparaginase, we have studied the circulatory properties of a dextran-asparaginase conjugate in patients with acute lymphoblastic leukaemia to assess its therapeutic potential.

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Materials

Erwinia carotovora asparaginase suitable for clinical use was purchased from British Drug Houses. Clinical dextran of 70000 daltons, dextran T fractions, Sepharose 4B and Sephadex G.25 were purchased from Pharmacia. Nesslers Reagent was prepared as described in Appendix 42 of the 1973 British Pharmacopoeia. Amino acid analysis was performed on a Chromaspek J180 amino acid analyser (Hilger Instruments, Margate, U.K.). Reagents used were of the purest grades

available and were purchased from British Drug Houses.

Methods

Preparation of the asparaginase conjugate

The procedures were carried out aseptically in the sterile environment of a positive pressure laminar flow cabinet. Samples (1·0 ml) of the conjugate were taken at suitable intervals during preparation and cultured in liquid media suitable for the detection of microbial contamination. No growth was observed.

50 ml clinical dextran (70 000 daltons, 6% w/v in 0·9% NaCl) was cooled to 4 °C on ice and slowly added to 3 g sodium metaperiodate dissolved in ice-cold distilled water (30 ml). The reaction was run overnight in the dark at 4 °C. The oxidized dextran was purified by passage over a sterile, calibrated Sephadex G.25 column (3 cm × 20 cm) equilibrated with 0·1 M potassium phosphate buffer pH 8·5. 100 mg asparaginase was reconstituted into 10 ml 0·1 M potassium phosphate buffer pH 7·0 and slowly added to 30 ml oxidized dextran, the two were stirred and left to react overnight at 4 °C in the dark. The conjugate was reduced by slowly adding 1 g $NaBH_4$ dissolved in ice-cold 10^{-3} M NaOH (5 ml), reduction was allowed to proceed for 2 h at 4 °C and then arrested by adding 1 M HCl to lower the pH to 7·0. The conjugate was purified by dialysis against 2 litres 0·9% w/v NaCl (Polyfusor Boots Co.) at 4 °C for 4 h and then by passage over a calibrated sterile Sephadex G.25 column (3 cm × 20 cm) equilibrated with 0·9% w/v NaCl. This procedure reduced phosphate levels to below 50 μg ml^{-1} indicating greater than 200 fold purification. The conjugate was sterilized by filtration (0·22 μm Millipore membrane), transferred to sterile injection bottles, shell frozen and aseptically freeze dried. Injections were stored at −40 °C until use whereupon they were reconstituted by addition of a suitable volume of water for injections.

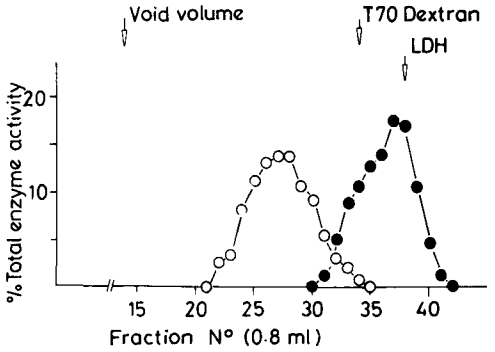

FIG. 1. Sepharose 4B exclusion chromatography of asparaginase (●) and the asparaginase-dextran conjugate (○). 0.5 ml samples of asparaginase (100 units) or the asparaginase conjugate (50 units) were eluted separately from the column (1·0 × 50 cm) using 0·1 M sodium phosphate buffer pH 7·0. The void volume was determined using blue dextran (2 × 10^6 daltons). Also shown are the elution volumes of lactate dehydrogenase and T70 dextran.

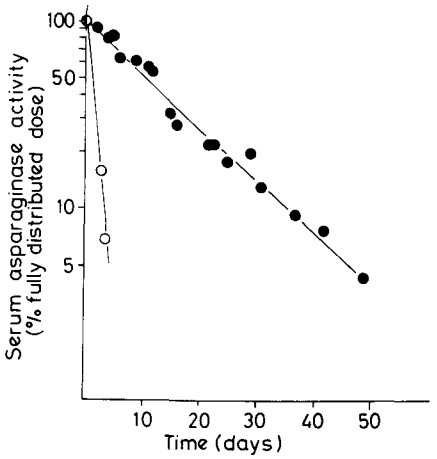

FIG. 2. Comparison of the circulatory properties of asparaginase (○) and the asparaginase-dextran conjugate (●). Patient A received an intravenous injection of native asparaginase (300 iu. kg^{-1}) and seven days later a similar dose of the asparaginase-dextran conjugate. The linear portion of each elimination curve was extrapolated to zero time to find the plasma level equivalent to the distributed dose. Plasma levels were then expressed as % of this value (approx. 7 i.u. ml^{-1}).

Determination of asparaginase and amino acids

Asparaginase activity was determined using the method of Wriston & Yellin (1973). Enzyme samples were added to 0·02 mmol L-asparagine dissolved in 2·0 ml 0·1 M sodium borate buffer at pH 8·5. The rate of ammonia production at 37 °C was determined using Nesslers Reagent. Preliminary tests showed that endogenous plasma asparaginase activity was absent.

To determine amino acid concentrations, blood samples (5·0 ml) were prevented from coagulation with lithium heparin and collected onto ice. They were immediately centrifuged (5000 rev min, 15 min, 4 °C) and the plasma supernatant immediately prepared for amino acid analysis by mixing with an equal volume (100 μl) of perchloric acid (10%) containing norleucine as internal standard (200 μmol $litre^{-1}$). After centrifugation the deproteinized supernatant was loaded onto the amino acid analyser for analysis using a standard 4 h salt loading program. The remaining plasma was immediately frozen at −20 °C before asparaginase activity measurement. Standard automated methods were used to assess liver and renal function.

Gel exclusion chromatography

Gel exclusion chromatography was carried out using columns of Sepharose 4B (1 cm × 50 cm). Samples (0·5 ml) were eluted in 0·1 M sodium phosphate buffer pH 7·0 containing 0·02% sodium azide. The void volume of the column was determined using blue dextran (2 × 10^6 daltons). The elution volume of lactate dehydrogenase was determined by analysing fractions for protein using the method of Lowry et al (1951) and

the elution of T70 dextran was followed using carbohydrate analysis (Dubois et al 1956).

Patient selection

Three patients, all of whom had relapsed lymphoblastic leukaemia and who were undergoing further remission induction therapy which logically would include asparaginase, were treated. One patient (A) gained a further partial remission and survived 14 months; the other two (B and C) had refractory disease and died 24 and 11 days after the start of the trial. All received one dose of the conjugate (300 i.u. kg^{-1}); none showed hypersensitivity reactions though patients A and B developed a metabolic acidosis. Serum samples taken before the start of the trial were negative when tested for the presence of asparaginase-specific antibody.

Results

Nature of the asparaginase-dextran conjugate

Asparaginase retained approximately 50% of its catalytic activity after conjugation to dextran. The elution profiles of free asparaginase and the reduced conjugate following Sepharose 4B exclusion chromatography are shown in Fig. 1. The conjugate eluted over a narrow molecular weight range indicating a homogeneous reaction product; moreover, very little enzyme activity eluted in fractions associated with the native enzyme, demonstrating that complete modification of asparaginase had taken place. The elution of the conjugate relative to the standards lactate dehydrogenase (Stokes' radius 4 nm) and T70 dextran (Stokes' radius 5·8 nm) showed that the conjugate was a large molecule with an approximate Stokes' radius of 10 nm.

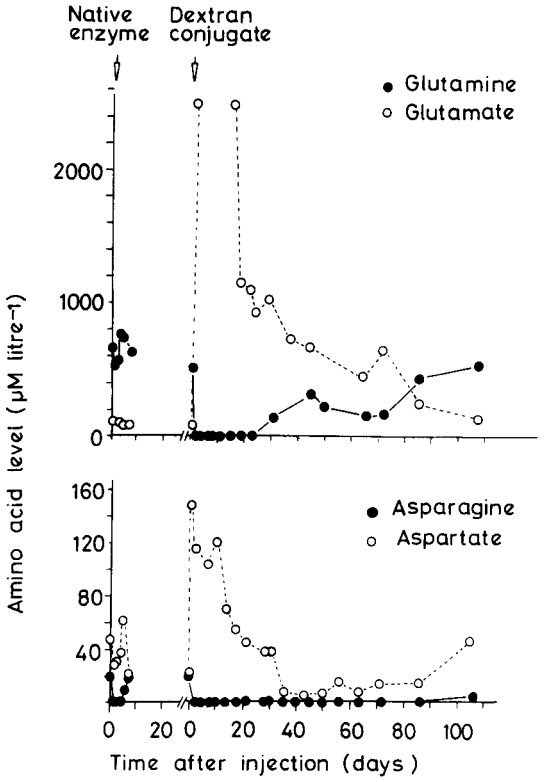

FIG. 3. Plasma amino acid concentrations following enzyme injections. Plasma samples from patient A were analysed for asparagine, aspartate, glutamine and glutamate as decribed in the text. Plasma asparaginase activities during this period are shown in Fig. 2.

Plasma asparaginase and amino acid concentrations

Fig. 2 compares the circulatory properties of native asparaginase and the enzyme-dextran conjugate in patient A. Unmodified asparaginase was rapidly removed and a half-life of 12 h was calculated. This result is in agreement with studies by Ohnuma et al (1972), who demonstrated plasma half-lives of between 7 and 13 h for *Erwinia* asparaginase in man. One week later the conjugate was tested; it survived much longer in the plasma and was eliminated by a single first order process with a half-life of 11 days.

Consistent with these results were studies of plasma amino acid concentrations (Fig. 3). Injection of the native enzyme caused a sharp fall in plasma asparagine which remained undetectable for five days, during which there was a small rise in plasma aspartate. A similar dose of the enzyme conjugate caused a profound change in amino acid concentrations. Plasma asparagine remained undetectable for 12 weeks and initially aspartate concentrations were raised threefold but returned to normal within three weeks. They then fell, probably as a result of chronic asparagine depletion since they rose to normal on the reappearance of asparagine in plasma samples. *Erwinia carotovora* asparaginase has a weak glutaminase activity (Howard & Carpenter 1972). The native enzyme caused a brief but probably insignificant fall in glutamine and did not affect glutamate, and amino acid concentrations were normal five days after injection. In contrast, the asparaginase conjugate caused a sharp fall of glutamine to undetectable levels which persisted for three weeks. During this period there was a sharp rise in glutamate values which started to fall on the reappearance of glutamine in plasma samples, and a further nine weeks passed before amino acid values returned to normal; glutamine recovery preceded asparagine recovery. Plasma albumin fell from 42 g $litre^{-1}$ on day 1 of the trial to 25 g $litre^{-1}$ on day 25, suggesting that chronic depletion of amino acids may have severely reduced albumin synthesis. Hypoalbuminaemia, as with conventional asparaginase therapy (Capizzi & Cheng 1981), was reversible and plasma albumin concentrations returned to normal on the return of normal plasma glutamine. Liver and renal function tests were normal throughout.

The conjugate was tested in two more patients (B and C). Their critical condition did not allow as detailed an analysis of enzyme and amino acid values, however, qualitatively similar results were recorded. In patient B

a plasma half-life of 7 days was calculated after observing elimination for 3 weeks; during which asparagine could not be detected in plasma samples. In patient C a half-life of 6 days was calculated from data collected over 5 days, asparagine remained undetectable during the trial. Marked glutaminase effects were observed in both patients, lasting for 11 days in patient B.

Discussion

Effective-asparaginase therapy producing continuous depletion of plasma asparagine requires daily or twice weekly intravenous injections of enzyme over two to four weeks (Capizzi & Cheng 1981). Animal studies have shown that polyethylene glycol (Bendich et al 1982), poly-D-alanyl peptide (Uren & Ragin 1979) and homologous albumin (Poznansky et al 1982) conjugates show enhanced circulatory survival. The long-term effects of these polymers in man, remain to be determined. We chose dextran in preference to these polymers because of its low toxicity as demonstrated during its use as a plasma expander in man.

We have shown that conjugation of asparaginase to clinical dextran of 70 000 daltons increases the plasma half-life of the enzyme approximately 20 fold so that in one patient the conjugate was able to deplete plasma asparagine for much longer (100 days) than a comparable dose of unmodified enzyme (seven days). These results suggest that a single dose of the conjugate could replace the repeated doses of asparaginase required at present. This would minimize the risk of a hypersensitivity reaction, for example life-threatening anaphylaxis, developing in response to the enzyme, and be more convenient for the patient.

The enhanced glutaminase activity shown by the conjugate probably results from the increased plasma persistence of the enzyme. Prolonged depletion of glutamine could be an advantage because glutamine, like asparagine, is essential for the growth of some tumours (Schrek et al 1971), and a glutaminase/asparaginase has undergone clinical tests (Spiers & Wade 1976). Unfortunately, glutamine depletion may have more profound metabolic consequences than asparagine removal (Harrison & Lilleyman 1978). In view of this, it should be emphasized that the material described herein is of an experimental nature and that formal phase II studies have yet to come. We cannot recommend its use in patients with leukaemia until a further assessment of the conjugate, particularly its glutaminase activity, has been made.

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