J. Pharm. Pharmacol. 1986, 38: 264–271
Received October 4, 1985

Soluble asparaginase-dextran conjugates show increased circulatory persistence and lowered antigen reactivity

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Oxidized dextrans of increasing molecular weight were bound covalently to *Erwinia carotovora* asparaginase. The resulting conjugates retained 50% of their enzyme activity and showed marked resistance to proteolysis by trypsin and chymotrypsin and inactivation by asparaginase-specific antibody. When tested in-vivo, the larger molecular weight conjugates showed prolonged circulatory survival in both immune and non-immune animals and failed to elicit full type III hypersensitivity or anaphylactic reactions when injected into sensitized guinea-pigs. Rabbits could tolerate multiple doses of the asparaginase conjugate without developing an immunity to the enzyme. A conjugate showing increased circulatory half-life and lowered antigen reactivity should have therapeutic potential.

The enzyme asparaginase isolated from *Erwinia carotovora* is used regularly in the treatment of acute lymphoblastic leukemias (Capizzi et al 1970). After injection the enzyme quickly depletes the serum of asparagine which is an essential nutrient for sensitive tumours (Broome 1961). Unfortunately, the relatively rapid elimination of asparaginase from the circulation means that a therapeutic response can only be achieved after repeated doses of the enzyme. Many attempts have been made to increase the circulatory survival of asparaginase, and Blazek & Benbough (1981) and Nickle et al (1982) were able to enhance the plasma persistence of microbial asparaginases by chemical modification. An alternative approach has involved binding the enzyme to soluble biocompatible polymers such as homologous albumin (Poznansky et al 1982), polyethylene glycol (Bendich et al 1982) or poly-D-alanyl peptides (Uren & Ragin 1979). These methods also have the advantage of sterically protecting the enzyme from immune recognition but unfortunately result in extensive loss of enzyme activity. This, and the fact that the biological effects of prolonged administration of polyethylene glycol and poly-D-alanyl peptides are at present unknown, has limited the therapeutic application of these conjugates. Dextran is used routinely as a plasma expander in man and its low toxicity is well established. Asparaginase may be bound to oxidized dextran without extensive loss of enzyme activity and asparaginase-dextran conjugates show prolonged circulatory half-lives in animals (Benbough et al 1979; Foster & Wileman 1979) and in patients with acute lymphoblastic leukemia (Wileman et al 1983).

Although the problem of short circulatory survival seems to have been overcome, immunological reactions to asparaginase remain a major limitation to its use. High circulating antibody titres cause rapid clearance of the enzyme and repeated injections are required to maintain adequate plasma levels. Hypersensitivity reactions, such as serum sickness and systemic anaphylaxis, may be life-threatening and require treatment to be stopped (Dellinger & Maile 1976). Our goal is to produce an asparaginase conjugate that combines the properties of prolonged circulatory survival reported previously (Wileman et al 1983) with lowered antigen reactivity. Such a formulation should have overcome the major limitations to the use of asparaginase in the treatment of leukemia. Conjugation of asparaginase to dextran should reduce the interaction between asparaginase and asparaginase-specific antibody and slow the elimination of the enzyme from the circulation of immune patients and help prevent the development of hypersensitivity reactions to the protein. In this study we have used the periodate oxidation method to bind asparaginase to dextrans of different molecular weights and have compared native asparaginase and the conjugates in a range of tests of antigen reactivity and immunogenicity.

MATERIALS AND METHODS

Oxidation of dextran and preparation of asparaginase conjugates

Polyaldehyde dextrans of mol. wt 10, 40, 70 and 250 kilodaltons were prepared as described previously

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(Wileman et al 1983). The aldehyde content of the polymer (600 mg) was determined using hydroxylamine reagent (British Pharmacopoeia 1973). Hydroxylamine hydrochloride reacts stoichiometrically with aldehydes to yield hydrochloric acid; the production of acid is determined by volumetric titration using potassium hydroxide. Modified dextrans were conjugated to *Erwinia carotovora* asparaginase as described by Benbough et al (1979). In addition, a batch of polyaldehyde dextrans of each molecular weight was reduced by sodium borohydride (10 mg ml^{-1} in 10^{-3} M NaOH) before addition of asparaginase. This provided a control to account for any effect that the unbound dextrans may have on the systems tested. Gel filtration analysis and asparaginase activity measurements were carried out as described (Wileman et al 1983).

Stability studies

Proteolysis of asparaginase and the dextran conjugates was carried out at 37 °C by incubation with trypsin or chymotrypsin (Sigma Chemical Co., Kingston, Surrey, UK) in 0·5 M sodium barbitone buffer, pH 8·0, containing 0·02 M $CaCl_2$. Each reaction mixture contained 25 μg ml^{-1} asparaginase and 10 μg ml^{-1} protease.

Immunization of rabbits

Asparaginase-specific antiserum was raised in male New Zealand White rabbits. The animals were injected intramuscularly with 500 μg *Erwinia carotovora* asparaginase emulsified in incomplete Freund's adjuvant (500 μl). The injection was repeated without the use of adjuvant 10, 20 and 40 days later. Serum samples taken 2 and 3 weeks after the last antigen challenge were pooled. Using immunoelectrophoresis, the precipitating antibody present was shown to be predominantly IgG.

Immunization of guinea-pigs

Male and female guinea-pigs were injected intramuscularly with *Erwinia carotovora* asparaginase (250 μg) dissolved in phosphate-buffered saline (100 μl) emulsified in an equal volume of Freund's incomplete adjuvant. The injections were repeated 14 and 28 days later without the use of adjuvant. Two weeks later the animals were tested for sensitivity by intradermal injections of the native enzyme (100 μg). Those animals showing a positive response were used in the experiment.

Immunoprecipitation

Quantitative immunoprecipitation from solution was carried out as described by Kabat (1961). Increasing quantities of rabbit antiserum were added to 100 μg antigen protein in a total volume of 600 μl phosphate-buffered saline. Precipitation was initiated by incubation at 37 °C for 2 h and followed by overnight storage at 4° C. Precipitation mixtures were resuspended and assayed for total asparaginase activity; precipitates were then pelleted by centrifugation (5 min, Beckman Microfuge), washed twice in phosphate-buffered saline (400 μl) and assayed for protein. Supernatants were tested for excess antibody or antigen by agar diffusion. Appropriate control tubes containing serum from a non-immunized rabbit were set up to detect non-specific effects of serum proteins on asparaginase activity. Tubes containing native asparaginase contained 60 mg reduced polyaldehyde dextran T70 for each mg of enzyme present, to control for the effects of non-attached dextran on immunoprecipitation and enzyme inactivation.

Circulatory survival of asparaginase and conjugate

The circulatory survival of asparaginase and the conjugates was tested using male New Zealand White rabbits (2–3 kg). The enzyme solutions were dialysed against 0·9% NaCl and sterilized by filtration (0·22 μm Millipore membrane) and then slowly injected into the marginal ear vein. Blood was collected from the corresponding vein in the other ear for measurement of enzyme activity. Solutions containing the native enzyme contained 60 mg of reduced polyaldehyde dextran T70 for each mg of enzyme to control for any effects of unattached dextran on enzyme elimination. Circulatory half-lives were calculated by least squares analysis of semi-logarithmic plots of serum enzyme activity versus time.

Arthus reaction of sensitized guinea-pigs

Six female guinea-pigs were sensitized as described above. Four weeks after the last sensitizing injection the hair was removed from the flanks by means of fur clippers and the remaining stubble by a depilatory cream. Each animal was injected intravenously with Evans Blue solution (0·5 ml 0·5% w/v). One hour later the antigen solutions equivalent to 100 μg protein were given by randomized intradermal injection into the exposed skin as were control solutions containing reduced polyaldehyde dextran T70 (60 mg ml^{-1}) of saline. Three such injections were administered to each flank allowing the native enzyme, the T10, T40 and T70 asparaginase conjugates and the control solutions to be studied in each animal. The native enzyme solution contained 60 mg

reduced polyaldehyde dextran T70 for each mg enzyme present.

Five hours following intradermal injection the guinea-pigs were studied by impartial observers for localized tissue damage, the intensity of each reaction being scored using a one to five rating scale (1, pale oedema localized within 0·5 cm of injection site; 2, pale oedema extending beyond 0·5 cm from injection site; 3, as for 2 but with dark oedematous centre; 4, as for 3 but with necrotic focus within 0·25 cm of injection site; 5, as for 3 but with necrotic focus extending beyond 0·25 cm from injection site). The reactions were further monitored after 24 and 72 h.

Dale-Shultz reaction

Eight guinea-pigs were sensitized to native asparaginase. Each animal was killed and the terminal 20 cm of ileum was removed and placed in oxygenated Ringer solution. The tissue was cut traversely into 2 cm lengths and, after removal of the attached mesentry, the tissue was suspended in a 10 ml isolated organ bath containing oxygenated Ringer solution at 37 °C and connected to an isometric strain gauge. The maximum contraction of the ileum to histamine was established first by constructing a dose response curve. The tissue was then washed and allowed to rest for 2 min and then challenged with antigen (400 μg protein). Tissue viability was then tested using a submaximal dose of histamine. The native enzyme (400 μg + 24 mg reduced polyaldehyde dextran) was then added as described above followed by the test dose of histamine. The contractile response of each ileal segment to the antigen was expressed as a percentage of that tissue's maximum response to histamine.

Anaphylaxis

Four guinea-pigs were sensitized to native asparaginase and then anaesthetized by intraperitoneal injection of pentobarbitone 40 mg kg^{-1}. The trachea of each animal was cannulated and connected to a pneumotachograph (A. Fleisch, Switzerland) connected to a Davis chart recorder. Two minutes after a steady breathing rate had been established, the dextran-T70 conjugate (equiv. to 1 mg protein) was injected intravenously via the femoral vein. The breathing rate was recorded for 3 min after which the animal was allowed to rest for 10 min and observed for respiratory distress. The second antigen (1 mg native enzyme +60 mg reduced polyaldehyde dextran T70) was then injected and breathing again monitored.

Immunogenicity

Control serum samples were taken from six rabbits (male, New Zealand White, 1·5 kg) and tested for asparaginase-specific antibody and shown to be negative. Three rabbits received weekly intravenous injections of a solution containing asparaginase (1 mg) and reduced polyaldehyde dextran T70 (60 mg). The other three rabbits received weekly intravenous injections of the T70 conjugate (1 mg protein). After each injection the serum half-lives of the enzyme or conjugate were determined and serum samples were taken one week later to test for the presence of antibodies able to precipitate asparaginase, asparaginase-T70 conjugate or reduced polyaldehyde dextran T70.

RESULTS

Polyaldehyde dextran

Polyaldehyde dextran was prepared by oxidation using sodium periodate, mild reaction conditions (low temperature and low periodate to glucose mole ratio) being chosen to limit overoxidation and consequent gross changes in the physicochemical properties of dextran. The elution profiles of T10, T40, T70, and T250 dextrans were compared before and after oxidation, and following reduction, using Sepharose 4B gel exclusion chromatography. The elution profiles of the dextrans were unaffected by chemical modification (results not shown) indicating that the hydrodynamic properties of dextran were not changed by oxidation or subsequent reduction. Reaction of dialysed polyaldehyde dextran with hydroxylamine reagent showed that 0·86 moles of aldehyde were introduced for each mole of glucose present in the reaction mixture. Since 2 moles of aldehyde would indicate complete oxidation we conclude that approximately 40% of the glucose residues were converted to the dialdehyde.

Dextran-asparaginase conjugates

Enzyme activity

Polyaldehyde dextran reacts with amino groups to form Schiff bases which were stabilized by reduction to secondary amines using sodium borohydride. Conjugation was routinely accompanied by a 50% retention of enzyme activity (250–350 iu mg^{-1}), the activity loss being independent of the molecular weight of the dextran bound to asparaginase. Specific asparaginase activities were determined at pH 8·5, the pH optimum for the enzyme. To determine the proportion of enzyme activity remaining at physiological pH, the assays were repeated at

pH 7·4. The native enzyme and the conjugates retained 90% of their maximum catalytic activity at physiological pH.

Gel exclusion studies

The efficiency of binding of dextran to asparaginase and the relative size and molecular weight distribution of the conjugates were studied using Sepharose 4B gel exclusion chromatography. The elution profiles of the reduced conjugate reaction mixtures are shown in Fig. 1. Little enzyme activity was eluted in

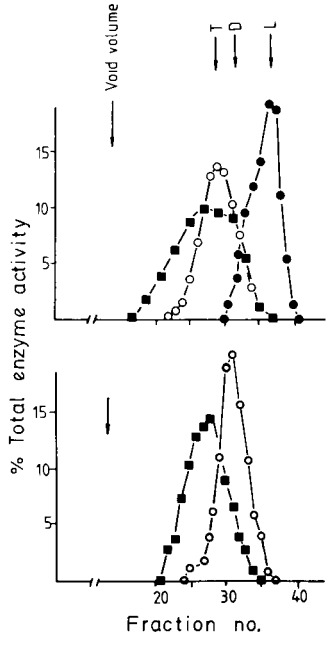

FIG. 1. Sepharose 4B chromatography of asparaginase and asparaginase conjugates. The top panel shows the elution profile of unmodified asparaginase 500 μg (mixed with 30 mg reduced polyaldehyde dextran T70) (●), and those of the reduced T40 (○) and T250 (■) conjugates. The lower panel shows the elution profiles of the reduced T10 (○) and T70, (■) conjugates of asparaginase. Also shown are the positions of peak elution of lactate dehydrogenase (L), T70 dextran (D) and thyroglobulin (T).

fractions associated with the native enzyme and, with the exception of the T250 conjugate, the reaction products eluted over a relatively narrow molecular weight range. The broader elution profile of the T250 conjugate may reflect the relatively broad molecular weight distribution of the parent dextran. Even though fractions containing peak enzyme activity eluted earlier from the column as the size of the dextran attached to the enzyme increased, there was a significant enough overlap in elution profiles to suggest that the sizes of the T250, T70 and T40 conjugates were similar. They were however significantly larger than the T10 conjugate. It was not possible to determine molecular weights accurately since suitable reference molecules containing both carbohydrate and protein were not available for column calibration. Their elution relative to lactate dehydrogenase, T70 dextran and thyroglobulin (Stokes radii of 4·1, 5·8 and 8·5 nm, respectively, Granath & Kvist 1953; Andrews 1970) nevertheless implied that they were large molecules varying in size between 7 nm (T10 conjugate) and 11 nm (T40, T70, T250 conjugates) radius.

Stability of the conjugates towards proteolysis

In this study asparaginase inactivation during incubation with proteases was used to assess the relative stabilities of the conjugates. They showed considerable resistance to trypsin (Fig. 2A) and stability increased with the size of the dextran attached. Native asparaginase and the conjugates were more sensitive to the action of chymotrypsin but the relative stabilities of the conjugates remained the same (Fig. 2B).

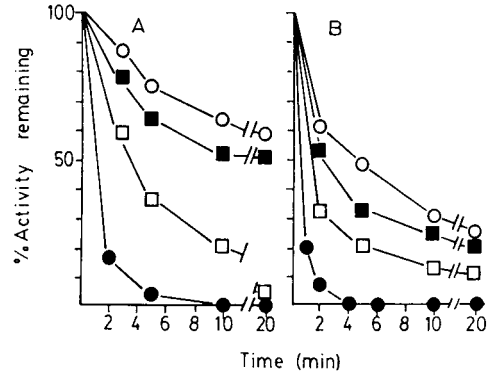

FIG. 2. Stability of asparaginase and asparaginase conjugates towards proteolysis. Asparaginase (50 μg) (mixed with 3 mg reduced polyaldehyde dextran T70) (●), or the reduced conjugates equivalent to 50 μg protein, (□) T10, (■) T70, (○) T250 were incubated at 37 °C with 20 μg of trypsin (Panel A), or chymotrypsin (Panel B). Total reaction volume = 2 ml.

Antigen reactivity of conjugates

The antigen reactivities of the asparaginase conjugates were compared using quantitative immunoprecipitation from solution. Fig. 3 shows that native asparaginase and the T10 conjugate, but not the T70 conjugate, readily precipitated from solution. Asparaginase activities remaining after incubation were determined (Fig. 4). A comparison of the volumes of antiserum required to inactivate 50% of the enzyme

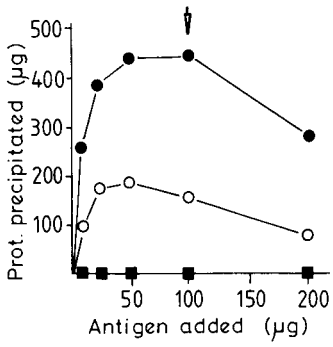

FIG. 3. Quantitative immunoprecipitation of asparaginase conjugates. Increasing quantities of rabbit antiserum were added to 100 µg asparaginase (mixed with 6 mg reduced polyaldehyde dextran T70) (●), or to the T10 (○) or T70 (■) conjugates (equivalent to 100 µg protein). After incubation, any precipitates were pelleted and assayed for protein, and supernatants were tested for antibody or antigen excess. The equivalence point is shown by the arrow. The T70 conjugate failed to precipitate.

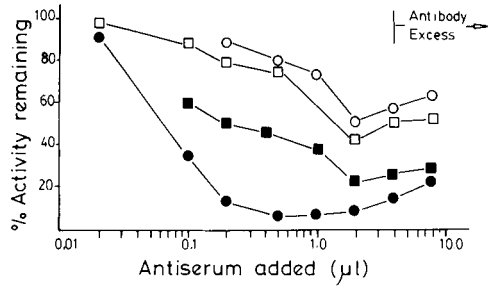

FIG. 4. Enzyme inactivation by asparaginase—specific antibody. Reaction mixtures were prepared as described in the legend to Fig. 3. After incubation the immunoprecipitates were suspended and a sample taken to determine total asparaginase activity remaining. This was expressed as a percentage of the activity added to the tube; (●) native asparaginase, (■) T10 conjugate, (□) T70 conjugate, (○) T250 conjugate.

provided an estimation of conjugate stability. Half the activity of native asparaginase was neutralized by addition of 0·05 µl of antiserum for each µg of protein present, similar inhibition of the T70 and T250 conjugates requiring 40-fold more antiserum (2 µl). The T10 conjugate was less stable requiring only 0·2 µl antiserum. The conjugates retained a residual activity in antibody excess, probably indicating a complete masking of some antigenic determinants, this activity increasing from 20% for the T10 conjugate to 50% for the T70 and T250 conjugates.

Circulatory survival of asparaginase conjugates in immune and non-immunized rabbits

The rate of elimination of native asparaginase in non-immunized rabbits was independent of dose between 80 and 600 iu kg^{-1}, and, after a brief period of tissue distribution (2 h), followed a single first order process with a half-life of 8 h (Table 1). The

Table 1. Circulatory properties of asparaginase and asparaginase conjugates in immune and non-immune rabbits. Non-immune rabbits received intravenous injections of asparaginase (300 iu kg^{-1}) or the respective asparaginase-dextran conjugate (300 iu kg^{-1}). Two hours after injection, a serum sample was taken and assayed for asparaginase activity; subsequent samples were taken at suitable intervals to follow the elimination of enzyme activity over at least two half-lives. Serum half-lives were determined by least squares analysis of semi-log plots of serum enzyme activity versus time. Three rabbits were used for each determination. In parentheses are the correlation coefficients for the first order elimination. Immune rabbits received a similar dose of enzyme or conjugate, but rapid elimination of the native enzyme or T10 conjugate precluded an accurate determination of circulatory half-life. Other half-lives were calculated as above. 60 mg ml^{-1} reduced polyaldehyde dextran T70 was added to native enzyme solutions (1 mg ml^{-1}) to control for non-specific effects of unattached dextran on enzyme elimination.

Enzyme	Mean plasma half-life (h) Non-immune		Immune	
Asparaginase	8·0	(0·99)	<0·1	—
T10 conjugate	8·3	(0·99)	<0·1	—
T40 conjugate	45·9	(0·98)	1·6	(0·99)
T70 conjugate	55·9	(0·98)	1·7	(0·95)
T250 conjugate	35·5	(0·98)	7·1	(0·95)

elimination profile of the T10 conjugate was the same as that of the native enzyme. In contrast, the T40, T70 and T250 conjugates survived for much longer in the plasma; they too were eliminated by single first order processes and half lives of 46, 56 and 36 h, respectively, were calculated. Both the native enzyme and the conjugates were eliminated rapidly from the plasma of immune rabbits, dextrans of 40 000 daltons or greater offering a significant degree of protection against antibody-mediated clearance.

Type III hypersensitivity in immunized guinea-pigs

Five hours after injection the responses to all three asparaginase conjugates were mutually indistinguishable (Table 2), the reactions being similar to those seen in response to dextran. Tissue damage in response to the enzyme conjugates and to dextran alone remained unchanged 24 h after injection and was fully reversed within 72 h (Table 2). In contrast, an area of necrosis developed at the site of injection of unmodified asparaginase, and this is reflected in the increased tissue damage rating seen at 24 h, necrosis failing to heal within 72 h.

Table 2. Tissue damage occurring during Arthus reaction of sensitized guinea-pigs. Tissue damage was visualized by an accumulation of Evans Blue dye at the site of injection. The extent of damage was assessed by impartial observers using a 1 to 5 rating scale. The points awarded to each antigen were totalled and expressed as a percentage of the maximum available points (25). Six animals were used to assess each antigen; mean responses ± standard error are shown in the Table.

Test solution	Mean response after 5 h (% ± s.e.)	Mean response after 24 h (% ± s.e.)
Saline	12·1 ± 2·0	2·2 ± 1·4
Dextran T70	25·5 ± 2·2	12·2 ± 3·9
Asparaginase	79·9 ± 7·0	92·2 ± 3·1
T10 conjugate	43·2 ± 4·0*	39·9 ± 3·8*
T40 conjugate	46·7 ± 4·1*	45·5 ± 2·1*
T70 conjugate	37·7 ± 5·5*	38·8 ± 4·6*

* Statistically significant difference from asparaginase.

Anaphylactic reactions

A strong tissue contraction resulted from incubation with the T10 conjugate (Fig. 5). Presumably this exhausted most of the antigen-releasable histamine

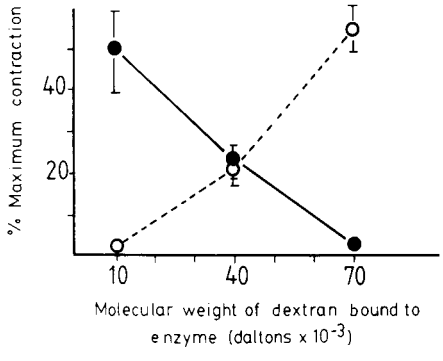

Fig. 5. Dale Schultz reaction: response of sensitized guinea-pig ileum to antigen solutions. Segments of sensitized guinea-pig ileum were incubated with asparaginase conjugate equivalent to 400 μg protein. The x axis shows the molecular weight of the dextran attached to asparaginase. The response of the tissue to the conjugate (●) (y axis) is expressed as a percentage of the tissue's maximum response to histamine. Residual histamine release in response to a subsequent challenge with 400 μg native asparaginase is also shown (○). This solution also contained 24 mg reduced polyaldehyde dextran T70. These data represent the mean of results from eight guinea-pigs (bars show standard errors).

since the tissue could respond only weakly to a subsequent challenge with asparaginase. The reverse was true for antigenic challenge with the T70 conjugate; the conjugate caused a weak contraction of the ileum which was then able to contract strongly on addition of unmodified asparaginase. The T40 conjugate was similarly tested, and the tissue's response to the conjugate and unmodified enzyme were approximately equal. In view of the low response of the sensitized ileum to the T70 conjugate, the effect of intravenous injection of the conjugate into sensitized guinea pigs was studied. Fig. 6 is an example of the results obtained from four

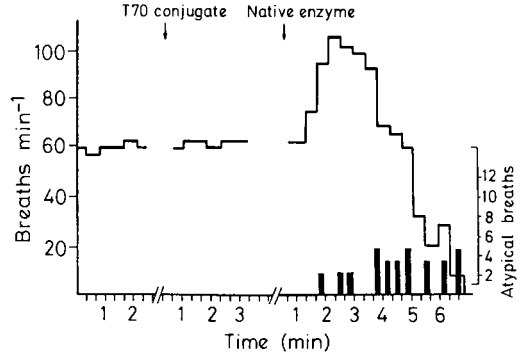

Fig. 6. In-vivo anaphylaxis: respiratory response of sensitized guinea-pigs to intravenous injection of antigen solutions. Guinea-pigs sensitized to asparaginase were given intravenous injections of the T70 asparaginase conjugate (1 mg protein). The graph is representative of results from four test animals. The y axis shows the breathing rate of the animal calculated from pneumotachograph recordings. The shaded bars show the number of atypical breaths occurring during each period. 10 min after injection of the T70 conjugate each animal received 1 mg asparaginase + 60 mg reduced polyaldehyde dextran.

test animals. Injection of the T70 conjugate had no significant effect on the breathing rate of the guinea-pigs. In contrast an injection of unmodified asparaginase caused a rapid rise in breathing rate and signs of respiratory distress. There then followed a sharp fall in breathing rate and death occurred within 5 min as a result of respiratory collapse.

Immunogenicity

The immunogenicities of asparaginase and the T70 conjugate following repeated intravenous injection into rabbits were compared. Fig. 7 shows that the unprotected enzyme gave rise to an immune response during the third week of dosing. This was characterized by a sharp fall in plasma half-life and the detection of an enzyme-specific antibody in serum samples. The plasma half-life of the T70 conjugate remained unchanged throughout the test period and the rabbits failed to produce an antibody able to precipitate asparaginase, dextran or reduced polyaldehyde dextran. Seven days after the last injection of the conjugate the plasma half-life of the native enzyme in the conjugate-treated rabbits was

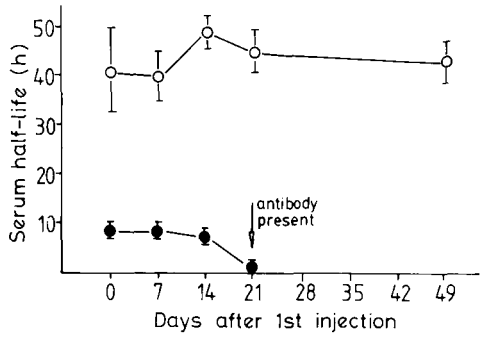

FIG. 7. Comparison of immunogenicity of asparaginase and asparaginase T70 conjugate. 3 male New Zealand White rabbits received either native asparaginase (1 mg + 60 mg reduced polyaldehyde dextran T70) (●) or the T70 conjugate equivalent to 1 mg protein (○), by intravenous injection. After each injection the serum half-life of the enzyme or conjugate were determined (y axis). One week later serum samples were taken and tested for the presence of cross-reacting antibodies. The injections were then repeated as shown.

determined and found to be the same as that seen in untreated animals (8 h, data not shown). These results are evidence that an immune response towards asparaginase had not been induced by repeated injections of the asparaginase T70 conjugate.

DISCUSSION

Gel exclusion studies showed that conjugation resulted in complete modification of the asparaginase present in the reaction mixture to produce molecules of high molecular weight. The T40, T70 and T250 conjugates were of similar size but larger than the T10 conjugate. The observation that the larger conjugates had similar hydrodynamic properties was surprising since the dextrans attached to the enzyme vary markedly in size. One explanation is that the size of the conjugate is limited by the steric packing of dextrans at the surface of the enzyme. Thus many T40 dextrans bound to asparaginase would increase the size of the conjugate in a manner comparable to the binding of only a few T250 dextrans.

Dextrans bound to asparaginase protect the enzyme from inactivation by proteases and enzyme-specific antibody and also reduce the antigen reactivity of the enzyme when tested in-vivo. This increased stability could have been effected through a number of mechanisms. Polyaldehyde dextran may have modified lysine residues present in the antigenic determinants of asparaginase or at trypsin binding sites. In addition, the dextran polymer may sterically hinder the interaction between asparaginase and these macromolecules. In many experiments stability increased with the size of dextran attached to the enzyme, an observation consistent with a role for steric hinderance, particularly since this relationship was observed when the conjugates were incubated with chymotrypsin, which, unlike trypsin, binds to aromatic amino acids rather than lysine residues.

The interactions between asparaginase and asparaginase-specific antibody has additional components. IgG is divalent and able to crosslink asparaginase into a precipitating immune complex. Precipitation may contribute to enzyme inactivation. This would appear unlikely because asparagine is of low molecular weight and easily able to permeate an immune complex (Cinader 1967), and in general immunoprecipitation inactivates enzymes acting on large substrates. Nevertheless, IgG was able to partially inactivate the T70 and T250 conjugates showing that IgG can move between the attached dextrans and bind to the enzymes surface. Interestingly enough, the antibody was unable to bring about precipitation of these conjugates. An explanation for this may lie in the fact that the diameters of these dextrans in solution are large (20 and 12 nm, respectively, Granath & Kvist 1953) when compared with the maximum distance between the F_{ab} antigen binding portions of IgG (14 nm, Feinstein & Beale 1977) and could conceivably prevent the antibody from spanning two dextran layers to bring about multivalent binding and subsequent precipitation (Fig. 8). An alternative explanation is that the

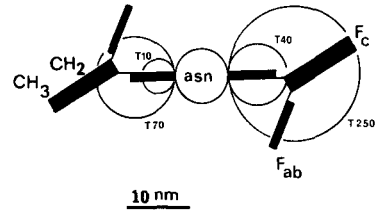

FIG. 8. Model for antibody binding to asparaginase-dextran conjugates. The diagram is drawn to scale assuming that the maximum distance between the F_{ab} binding sites is 16 nm (Fienstein & Beale 1977) and the diameters of T40, T70 and T250 dextrans in solution to be approximately 9, 12 and 20 nm, respectively (Granath & Kvist 1953). IgG can pass between dextran strands attached to the surface of asparaginase and bind to antigenic determinants associated with catalytic activity (see Fig. 4). Large dextrans (T40, T70, T250) inhibit the interaction between the enzyme-bound immunoglobulin and macromolecules such as (a) other antigens binding the F_{ab} region (see Fig. 3), (b) effector molecules of the complement cascade binding to the CH_2 domain of the F_c region (Table 2) or (c) binding of the CH_3 domain of the F_c portion to F_c receptors within the reticuloendothelial system (see Table 1). T10 dextran of 5 nm diameter would be expected to have little effect on the effector functions of enzyme-bound immunoglobulin.

hydrophilic dextran molecules increase the water solubility of the immune complexes and prevent precipitation. This would appear unlikely since dextran alone can precipitate an anti-dextran immunoglobulin from human immune serum (Kabat & Beezer 1958). The residual enzyme activity seen in antibody excess (Fig. 4) is a common feature of enzyme inactivation by antibody and has been studied in detail by Ng & Gregory (1969). It is attributed to mutual exclusion of antibodies from the surface of the enzyme. Presumably the increased residual activity shown by the conjugates reflects additional exclusion of inactivating antibodies from asparaginase by the attached dextran.

In general terms, antigen reactivity in-vivo fell with an increase in the size of the dextran attached to asparaginase. Because immune reactions follow complicated pathways the precise points of inhibition by the attached dextran are unknown. As described above, lowered antigen : antibody affinity and inhibition of immune complex formation are likely to play a role. Dextran may also shield the F_c portion of the enzyme-bound IgG (Fig. 8) and hinder subsequent binding of complement components, as occurs during the Arthus reaction, or reduce binding to the F_c receptors of the reticuloendothelial system responsible for immune clearance. The T70 conjugate was unable to induce an anaphylactic response suggesting that the dextran can also hinder the interaction between asparaginase and tissue-bound IgE.

The above studies suggested that stabilization of asparaginase was optimized by binding the enzyme to T70 dextran, a polymer used routinely as a plasma expander in man. The development of an immune response and the risk of systemic anaphylaxis are major limitations to the clinical use of asparaginase. Two results from this study are, therefore, particularly interesting. Firstly, intravenous injection of the T70 conjugate failed to induce an anaphylactic response in sensitized guinea pigs and secondly, repeated intravenous injection of the conjugates into rabbits using a protocol similar to that used during the clinical use of the enzyme failed to provoke an immune response to asparaginase or dextran. We have shown that asparaginase bound to clinical T70 dextran has a prolonged plasma half-life in man (Wileman et al 1983) and conclude that dextran-asparaginase conjugates have a therapeutic potential for the treatment of acute lymphoblastic leukemias.

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