Enhanced Plasma Persistence of Therapeutic Enzymes by Coupling to Soluble Dextran

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Conjugation of carboxypeptidase G and arginase, two enzymes of therapeutic interest, to a soluble dextran significantly enhanced plasma persistence in normal and tumour-bearing mice. A prolonged decrease in arginine concentrations in plasma of tumour-bearing mice was demonstrated by using the dextran-linked arginase. Gel filtration of dextran-enzyme conjugate showed that enzyme activity co-chromatographed as a single peak with carbohydrate, and enzyme was shown to be covalently linked to the dextran.

Microbial enzymes have a relatively brief persistence in mammalian circulation, their plasma half-lives ($t_{1/2}$) being almost always less than 10 h. This is a major limitation to the wider therapeutic use of these enzymes, and several methods of prolonging their persistence by chemical modification or by enclosure of the enzyme within liposomes (Neerunjun & Gregoriadis, 1976) and erythrocytes (Updike *et al.*, 1976) have been examined.

Vegarud & Christensen (1975) showed that various glycosidases and proteinases had increased stability towards thermal and proteolytic inactivation *in vitro* when coupled with polysaccharides, and heparin was shown to have a distinctly longer half-life *in vivo* when coupled to the sucrose polymer Ficoll (Pharmacia, Uppsala, Sweden) (Teien *et al.*, 1975). Holcenberg *et al.* (1975) also showed the increased $t_{1/2}$ of *Acinetobacter* glutaminase-asparaginase in mice and rats when the enzyme was modified by glycosylation with glycopeptides prepared from human fibrin and γ -globulin.

We have investigated the persistence of two enzymes coupled to a soluble dextran, which is used as a blood-volume expander and persists in the circulation for long periods ($t_{1/2}$ = 20–50 h) (Wilson *et al.*, 1952).

Materials and Methods

Glycosylation of enzymes

Carboxypeptidase G (EC 3.4.12.10) (dimer of subunits of mol.wt. 40000 containing bound Zn^{2+}), a folic acid-hydrolysing enzyme from a mutant of *Pseudomonas* sp. A.T.C.C. 25301, and an arginase (EC 3.5.3.1) (tetramer of subunits of mol.wt. 42000) from *Fusarium solani* I.M.I. 204449 were purified to homogeneity, as judged by sodium dodecyl sulphate/poly-

acrylamide-gel electrophoresis and coupled to a soluble dextran preparation (Lomodex 40; Fisons, Loughborough, Leics., U.K.) activated with CNBr.

Dextran solution (35 ml) containing 1 g of soluble dextran (approx. mol.wt. 40000) was diluted to 100 ml and three portions (0.2, 0.2 and 0.1 g) of CNBr were added at 10 min intervals. The vigorously stirred mixture was maintained at pH 10.5–11 by the addition of 2 M-NaOH, and approx. 30 min after the addition of the last portion of CNBr the solution was twice diluted 5-fold with water, and then was concentrated to the original volume (35 ml) by using an Amicon CH3 concentrator fitted with a 5000-dalton hollow-fibre cartridge. The activated dextran preparation was then added to carboxypeptidase G solution (8 ml, 320 units) in 0.1 M-Tris/acetic acid buffer, pH 7.3, or arginase solution (5 ml, 1280 units) in 0.05 M-sodium phosphate buffer, pH 8.0, containing 0.4 mM-MnCl₂, and the final solution (40 ml) stirred overnight at 4°C.

The dextran-coupled enzymes were fractionated by gel filtration on a column (92 cm × 5 cm diam.) of Sephadex G-150 (2 litres bed volume). Protein and carbohydrate contents of the eluted fractions (10 ml) were determined by the methods of Lowry *et al.* (1951) (bovine serum albumin standard) and Dubois *et al.* (1956) (Lomodex 40 dextran standard) respectively.

Enzyme assays

Carboxypeptidase G activity was assayed at 30°C in 0.1 M-Tris/HCl (pH 7.3)/0.06 mM-methotrexate/0.1 mM-ZnCl₂ in a final volume of 1 ml. The reaction was started by addition of enzyme and followed by the decrease in A_{320} (McCullough *et al.*, 1971). One unit of carboxypeptidase G was defined as that amount hydrolysing 1 μ mol of methotrexate/min at 30°C.

Arginase activity was assayed at 37°C in 0.05 M-sodium phosphate buffer (pH 8.0)/0.02 M-arginine/

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Fig. 1. Elution profiles of dextran-enzyme conjugate from a 2-litre Sephadex G-150 column

(a) Carboxypeptidase G was eluted with 0.05M-Tris/acetic acid buffer, pH 7.3, at 45 ml/h. Fractions 54-62 were pooled and the recovered enzyme (165 units) was concentrated on an Amicon B-15 ultrafilter to give a solution of 25.3 units/ml. (b) Arginase was eluted with 0.05M-sodium phosphate buffer, pH 8.0, containing 0.4mM-MnCl₂, at 40 ml/h. Fractions 44-52 were pooled and the recovered enzyme (550 units) was concentrated as above to give a solution of 24 units/ml. ●, Enzyme; ▲, carbohydrate; ○, protein.

0.4mM-MnCl₂ containing 0.1mg of bovine serum albumin/ml by using an autoanalyser system that determined the release of urea, based on the method of Archibald (1944). One unit of arginase was defined as that amount liberating 1 μmol of urea/min at 37°C.

Enzyme persistence in vivo

Four groups of four 6-week-old female DBA/2 mice (Olac, Bicester, Oxford, U.K.) were inoculated intraperitoneally with 10⁶ P815Y mastocytoma cells (Chou & Handschumacher, 1972). Four control

groups were given phosphate-buffered 0.9% NaCl. After 3 days each group was given intraperitoneally 1 ml of carboxypeptidase G (26.5 units of native enzyme or 25.3 units of the fractionated dextran-linked enzyme) or arginase (30 units of native enzyme or 24 units of dextran-linked enzyme). Blood samples (10 μ l) were taken from individual animals at intervals thereafter over a 4-day period and assayed for enzyme activity.

In a second experiment, 12 groups of three tumour-bearing mice were inoculated intraperitoneally with enzyme (30 units of native arginase or 36 units of dextran-arginase) and killed at intervals over a 2-day period. Blood samples were analysed for arginine by using a Locarte automatic amino acid analyser.

Results and Discussion

Kagedal & Akerstrom (1971) showed that treatment of soluble dextrans with CNBr gave activated dextrans with a lowered water-solubility, and isolation by precipitation with acetone gave only partially water-soluble products, presumably due to intermolecular cross-linking. It was found that treatment of the dextran with several small portions of CNBr and removal of the low-molecular-weight reaction products at the end of activation by repeated concentration and dilution by using an ultrafiltration system eliminated these problems. This isolation procedure also gave an activated dextran that was less inhibitory to the enzyme in the subsequent coupling step.

Chromatography of coupled carboxypeptidase on Sephadex G-150 (Fig. 1a) showed that the enzymic activity co-chromatographed as a single peak (maximum activity eluted at 580 ml) with both the carbohydrate and protein, which indicated that the dextran-enzyme conjugate had a mol.wt. of about 130000. There was no evidence of free native enzyme (mol.wt. 82000), which is eluted with a peak value at 840 ml. The activity of the dextran-carboxypeptidase conjugate represented a 52% recovery of the original enzyme activity, and the enzyme was shown to be covalently linked to the dextran by the precipitation of dextran-enzyme conjugate with concanavalin A (the free native enzyme is not precipitated with the latter even in the presence of added dextran solution) and the inability to separate the dextran-enzyme conjugate into free dextran and enzyme in the presence of 3M-NaCl or 6M-urea.

Chromatography of coupled arginase on Sephadex G-150 (Fig. 1b) also showed that enzyme activity co-chromatographed as a single peak with carbohydrate and protein, in this case at the void volume. The activity of the dextran-arginase conjugate was 45% of the original enzyme. Gel filtration of native arginase (mol.wt. 168000) and coupled arginase on a Sephadex G-200 column calibrated with catalase

Fig. 2. Elution profile of native arginase and dextran-arginase conjugate separated on a Sephadex G-200 column

Native arginase (●) (200 units) and dextran-arginase conjugate (○) (12 units) were loaded in 1 ml of 5% (v/v) glycerol solution on to a calibrated Sephadex G-200 column equilibrated in 0.05M-potassium phosphate buffer, pH 6.8, containing 1.4mM-mercaptoethanol. Also included in the sample were methionyl-tRNA synthetase (0.5mg, peak indicated M at 57ml), aldolase (2mg, peak indicated A at 61ml) and catalase (2mg, peak indicated C at 52ml) as marker proteins. The column was eluted (descending) with the same buffer at 4ml/h.

Table 1. Persistence of native enzyme and dextran-enzyme conjugates in normal and tumour-bearing DBA/2 mice. Half-lives ($t_{1/2}$) of the enzymes were calculated from semi-logarithmic plots of enzyme activity in the blood against time, and are shown as means $\pm$ s.d. of results from groups of three or four mice.

	$t_{1/2}$ (h)	
	Normal mice	Tumour-bearing mice
Native carboxypeptidase G	3.5 $\pm$ 0.5	7 $\pm$ 1.2
Dextran-carboxypeptidase G conjugate	17 $\pm$ 3.2	18 $\pm$ 2.0
Native arginase	1.4 $\pm$ 0.2	2.5 $\pm$ 0.3
Dextran-arginase conjugate	12 $\pm$ 1.7	17 $\pm$ 2.5

(mol.wt. 240000), methionyl-tRNA synthetase (mol.wt. 180000) and aldolase (mol.wt. 156000) as marker proteins showed that coupled enzyme was eluted at a position which indicated a mol.wt. of about 260000 (Fig. 2).

Molecular-weight data from the Sephadex columns indicated that 1 and 2 mol of dextran bound to 1 mol

of carboxypeptidase G and 1 mol of arginase respectively.

Experiments with mice showed that the clearance rates of the dextran-enzyme preparations from both normal and tumour-bearing animals were 2–3-fold slower (carboxypeptidase) and 6–8-fold slower (arginase) compared with tumour-bearing animals given the native enzymes and were considerably slower than normal animals given native enzyme (Table 1). The observation that the native enzymes are more rapidly cleared from normal mice than from those bearing a tumour, by a factor of approx. 2, has been found on several occasions (C. N. Wiblin, unpublished work). It is reminiscent of the lactate dehydrogenase virus-associated effects reported for some other microbial enzymes of chemotherapeutic value (Riley *et al.*, 1970, 1974). The complexed enzymes exhibit less marked variation in half-life, the values for tumour-bearing and normal animals being similar. Two other features of the conjugated enzymes *in vivo* were the apparent slower rate of adsorption from the peritoneal cavity and the higher concentrations reached in the plasma. Both are probably sequelae of the much decreased clearance rate and the latter is of obvious chemotherapeutic consequence.

The Michaelis constants (K_m) of carboxypeptidase G for various folate derivatives are low ($<10\mu\text{M}$) and comparatively small amounts would be required to deplete total plasma folate. However, it is expected that enzyme activity would have to be maintained for long periods to ensure depletion of the larger folate pool present in the liver. The use of the conjugated carboxypeptidase with its initially greater uptake and longer half-life would facilitate removal of this pool without the necessity of administering very high doses at frequent intervals with the risks that such schedules may involve.

A similar advantage is seen with conjugated arginase. The arginase from *F. solani* provided a good model for demonstrating the advantages of glycosylation, because of the extremely rapid clearance of native enzyme from the plasma. In addition the K_m for arginine is high (14 mM), and therefore of necessity the enzyme would have to be maintained in the plasma for long periods to ensure removal of arginine. Arginine analyses on blood samples from tumour-bearing mice treated with native enzyme showed only a transient lowering in concentration 1–2 h after administration of the enzyme; whereas with the dextran-arginase-treated animals arginine concentrations were still decreased after 13 h (Table 2). The incomplete removal of arginine (about 50%) was probably a reflection of the high K_m of the enzyme.

Covalent attachment of enzymes to soluble dextran provides a straightforward and rapid method of glycosylation. If increased persistence of enzymes *in vivo* upon glycosylation proves to be a general

Table 2. Depletion of arginine in tumour-bearing DBA/2 mice treated with native arginase and dextran-arginase conjugate

Groups of three mice were killed at intervals after intraperitoneal injection of enzyme (30 units of native arginase or 36 units of dextran-arginase per mouse) and blood was analysed for arginine. Results are expressed as means $\pm$ S.D. for each group.

Time (h)	Arginine concn. in blood (μM)	
	Native arginase	Dextran-arginase conjugate
0	134 $\pm$ 19	134 $\pm$ 19
1	96 $\pm$ 24	—
2	108 $\pm$ 10	107 $\pm$ 12
4	152 $\pm$ 13	93 $\pm$ 11
6	—	77 $\pm$ 17
8	—	103 $\pm$ 5
13	—	92 $\pm$ 10
24	140 $\pm$ 15	169 $\pm$ 17
48	—	153 $\pm$ 27

phenomenon, then it may be possible to increase the therapeutic efficiency of other enzymes at present being used as anti-tumour agents.

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