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COVALENT LINKAGE OF CARBOXYPEPTIDASE G_2 TO SOLUBLE DEXTRANS—I

PROPERTIES OF CONJUGATES AND EFFECTS ON PLASMA PERSISTENCE IN MICE

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Abstract—The covalent attachment of the therapeutic enzyme carboxypeptidase G_2 to soluble dextrans of varying molecular weight resulted in a 5–15-fold increase in plasma persistence in normal and tumour-bearing mice. The molecular weight of the dextran used markedly affected the number of dextran molecules present in the conjugate, resulting in a molecular weight distribution between 6 and 12×10^5 daltons. The isoelectric point of the conjugates varied between 4.1 and 4.8 compared to native enzyme 7.8. Conjugates were resistant to proteolysis by trypsin and chymotrypsin, but showed little difference in their affinity for substrate.

Microbial enzymes offer several possibilities for application in cancer chemotherapy. Continuous depletion of specific amino acids from body fluids by enzymatic action has proved to be a therapeutic approach demonstrating a high degree of specificity for the neoplasm. The enzyme asparaginase [1] has led the way in this field, but the effects of several other amino acid degrading enzymes have been described, notably glutaminase [2] and methioninase [3]. In a related manner, enzymatic depletion of the "folate pool" using enzymes of the class carboxypeptidase G can be used to inhibit tumour development [4, 5]. In practice the usefulness of an enzyme is often limited by its relatively short persistence in the blood, typically displaying half-lives of only 2–4 hr [6]. Various techniques for prolonging the persistence of such enzymes by chemical modification have been developed, including the covalent attachment of the enzymes to soluble dextran [6–9].

The mechanism of the enhanced plasma persistence afforded by the dextran carrier is not known. It has been postulated that the effect may be due to protection against degradation or removal of the intact protein through changes in the electrostatic charge of the conjugated protein [10, 11], or the increase in relative mass [12]. It is unlikely, however, that persistence is related in a simple fashion to such factors; recognition of "foreign" proteins and interaction with other blood components will form part of a complex clearance process. Conjugated enzymes have been shown to exhibit reduced immunogenicity when compared with native enzyme [13, 14] and also enhanced resistance to proteolysis and heat denaturation [15–17].

The soluble dextrans employed for coupling are themselves biologically inert, and are used clinically as blood volume expanders in a range of molecular weights, usually 40,000 to 150,000 daltons. Half-life in circulation varies from 6 to 50 hr for dextrans within this range [18, 19]. In view of these extended and varied half-lives, the possibility arises of increasing the persistence of therapeutic enzymes and to some extent controlling the improvement by choice of different molecular weight dextrans as carrier. In this paper we describe the preparation of conjugates of the folate degrading enzyme carboxypeptidase G_2 (CPG_2) [20], to dextrans having molecular weights of 40, 70, 110 and 150,000 daltons. We have investigated the properties of such conjugates and their circulation half-lives in mice.

MATERIALS AND METHODS

Enzyme assay. CPG_2 is a folic acid-hydrolysing enzyme isolated from a mutant of *Pseudomonas* sp. strain RS-16. The enzyme, a dimer with subunits of molecular weight 41,800 daltons, containing bound Zn^{2+} ions was purified to homogeneity as judged by sodium dodecylsulphate-polyacrylamide gel electrophoresis [20]. CPG_2 activity was assayed at 37° in 0.1 M Tris–HCl, pH 7.3 with 0.06 mM methotrexate (Lederle, Gosport, Hants., U.K.) as substrate and 0.2 mM $ZnSO_4$ in a total volume of 1 ml. The reaction was started by the addition of enzyme and followed by decrease in A_{320} [21]. One unit (U) of enzyme activity catalysed the conversion of 1 μmole methotrexate/min at 37°.

Glycosylation of CPG_2. CPG_2 was coupled to soluble dextrans (Lomodex 40, Lomodex 70, Dextraven 110 and Dextraven 150; Fisons, Loughborough, Leics., U.K.). The volume of dextran preparation containing 1 g of dextran in 0.9% NaCl was diluted to 100 ml with 0.9% NaCl and reacted with cyanogen bromide (Sigma, Poole, Dorset, U.K.). CNBr (0.5 g) was used for activating the 40- and 70,000 dalton dextrans and 0.4 g for the higher molecular weight

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dextrans. This reduction was necessary to prevent precipitation of the 110,000 and 150,000 dalton dextrans. The reaction mixture was vigorously stirred at room temperature and maintained at pH 10.7 ± 0.1 units in a pH-stat (Radiometer, Copenhagen, Denmark) by addition of 2 M NaOH. The CNBr was added as a finely divided powder in two equal portions at an interval of 20 min; the second portion was allowed to react until the pH of the reaction mixture was stable at 10.7; the pH was then adjusted to 9.0 and the mixture dialysed against running water for 2 hr at 4°. The pH was brought back to 9.0 with 1 M NaOH and 1 ml enzyme solution (1265 U; 2.3 mg) in 0.1 M Tris–HCl buffer, pH 7.3, added. The mixture was reacted overnight at 4° after which 0.25 g glycine was added to block excess reactive sites. The mixture was stirred for a further 30 min and then concentrated to a volume of 40 ml in a model 202 concentrator using a PM10 ultrafiltration membrane (Amicon, Stonehouse, U.K.). The mixture (40 ml) was then chromatographed on a 1.3-litre bed volume of Sephadex G150 in a 4.4 × 87 cm column (Pharmacia, Uppsala, Sweden) and eluted with 0.05 M potassium phosphate buffer, pH 7.0. fractions (10 ml) were collected and assayed for enzyme activity; carbohydrate content was determined by the phenol–sulphuric acid method [22] using dextran-70 as standard in the range 0–100 μg/ml.

The peak fractions were pooled and concentrated to a volume of 10–12 ml as before. Enzyme activity and carbohydrate content were determined and protein content measured by the Coomassie blue method [23] using bovine serum albumin fraction V as standard in the range 0–100 μg/ml. The concentrated material was filter sterilized (Millipore "Millex GS", 0.22 M pore size) and stored at −20°.

Preparation of ^{3}H-labelled dextrans. One gram of each dextran was activated as described. Immediately after activation 100 μCi (0.1 ml, 0.5 mg) of ^{3}H-glycine (New England Nuclear, Dreiech, F.R.G.) was added and the mixture incubated overnight. A further 0.25 g cold glycine was added and the mixture stirred for 30 min followed by concentration to a volume of 40 ml. The concentrated mixture was chromatographed on a 4.4 × 87 cm column of Sephadex G150 as described above. Fractions (10 ml) were collected and samples assayed for carbohydrate content and radioactivity, using 5 ml of "Aqualuma" scintillant fluid (LKB, Bromma, Sweden) and counted in an LKB model 1215 "Rackbeta" liquid scintillation counter equipped with a dpm calculation package. The peak fractions were pooled, concentrated and filter sterilized as described previously and stored at −20°.

Estimation of molecular weight of dextran–CPG_2 conjugates by gel filtration. The molecular weight of dextran–CPG_2 conjugates was estimated by descending analytical gel filtration on Sephacryl S400 (Pharmacia, Uppsala, Sweden) or Ultrogel AcA22 (LKB, Bromma, Sweden). Columns (2.2 × 87 cm) were packed according to the manufacturer's instructions and equilibrated with phosphate buffered saline (PBS = g/litre: NaCl, 8.0; KCl 0.2; Na_2HPO_4, 1.13; KH_2PO_4, 0.2; pH 7.4) at a flow rate of 20 ml/hr. Samples (2.5 ml) were applied directly to the surface of the gel, and 2.36 ml fractions were collected. Calibration curves using both protein and dextran standards were prepared and the molecular weight of the dextran–CPG_2 conjugates estimated by interpolation.

Estimation of the degree of binding of dextran to CPG_2 by competitive binding with glycine. Dextrans were activated as previously described. Upon completion of dialysis two aliquots of 10 ml were withdrawn from each sample of activated dextran. To one sample was added either CPG_2 (4.4 mg, 5.3×10^{-8} mol) or 1,6-diaminohexane (0.2 mg 1.72×10^{-6} mol) whilst the remaining aliquot was untreated. Samples (1 ml) were withdrawn after 24 hr and mixed with a solution of glycine (100 μl, 0.22 mg, containing 0.01% U-^{14}C-glycine (8.85×10^{-9} mol/μCi) (Amersham International, Amersham, Bucks., U.K.). The mixture was allowed to react for 1 hr at room temperature after which unbound glycine was separated by gel filtration on a pre-packed (PD10) column of Sephadex G25 (Pharmacia, Uppsala, Sweden), following the manufacturer's instructions. Aliquots (35 μl) of the desalted samples (3.5 ml) were mixed with 5 ml of "Aqualuma" scintillant fluid (LKB, Bromma, Sweden) and counted in an LKB "Rackbeta" model 1215 scintillation counter equipped with a dpm calculation program.

Determination of the resistance of CPG_2 and dextran–CPG_2 conjugates to proteolysis. Samples (1 ml) of CPG_2, dextran 70-CPG_2, or dextran 150-CPG_2 containing 100 μg protein in 0.05 M ammonium bicarbonate were reacted with either 10 μg of α-chymotrypsin of 5 μg of trypsin (Millipore Corporation, Freehold, NJ) added as 5 μl of a stock solution of 2 mg/ml and 1 mg/ml in 1 mM HCl respectively. Dextran 70 (10 mg) was added to the unmodified CPG_2 sample to compensate for the possible presence of unbound dextran in the dextran–CPG_2 conjugate samples. The reaction mixtures were incubated at 37° and 10 μl samples withdrawn at 10 min intervals for the determination of CPG_2 activity.

The effect of pH on the stability of CPG_2 and dextran–CPG_2 conjugates. The effect of pH on the activity of CPG_2 and dextran–CPG_2 conjugates was measured using the following buffers: pH 4.5–6.0, 0.1 M sodium acetate; pH 6.0–8.0, 0.1 M potassium phosphate; pH 8.0–10.5, 0.1 M borate/NaOH. Assay mixtures consisted of 0.9 ml buffer plus 0.1 ml methotrexate solution (0.6 mM in 0.1 M Tris–HCl, pH 7.3). One hundred-microlitre samples of stock solutions of CPG_2 or dextran–CPG_2 conjugates were assayed. Activities were calculated as the percentage residual activity, taking the activity in the standard assay mix as 100%.

Isoelectric focusing of dextran–CPG_2 conjugates. Agrose gels (1% w/v) (0.5 mm thick), containing 15% (v/v) pH 3.5–10.0 ampholines (LKB, Bromma, Sweden), and measuring 100 mm × 90 mm were cast on "Gelbond" film (FMC Corporation, Rockland, M). Samples (15 μl, containing 0.2–0.55 mg protein/ml) were loaded onto the longitudinal centre of the gel on filter paper measuring 5 mm × 10 mm. The gels were run horizontally in an LKB "Multiphor" apparatus for 1.5 hr at a constant power setting of 4.0 W and stained with Coomassie blue R250.

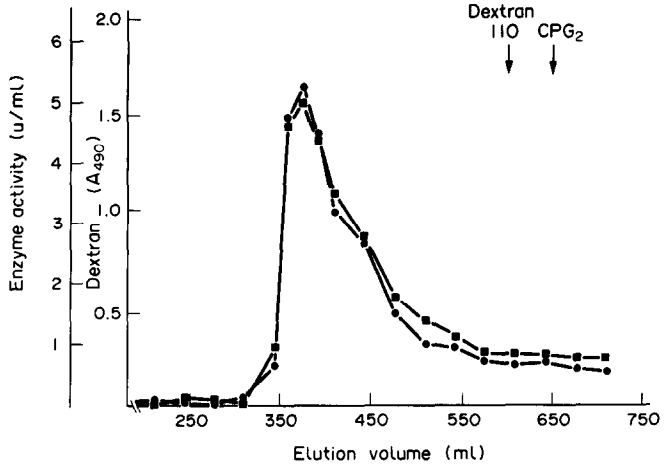

Fig. 1. Elution profile of dextraven (110) (■) and enzyme activity (●) from a 1.32 litre column of Sephadex G150; 10 ml fractions were collected and fractions 34–45 pooled and concentrated to give a solution of 33.7 U/ml. Elution was with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 40 ml/hr. Arrows mark the elution positions of native dextran 110 (610 ml) and CPG_2 (650 ml).

Enzyme persistence in vivo. Groups of 5 female Balb/c mice, 15–20 g in weight (Olac, Bicester, Oxon, U.K.) were used for persistence trials. Mice were injected intraperitoneally with 10^6 Ehrlich tumour cells (tumour bearing group) or PBS (normal group). After 72 hr each group was injected intravenously via the tail vein with 1 ml (25U) of each CPG_2 conjugate or with native enzyme. Animals injected with ^3H dextran samples received 1 ml solution, equal to 20 mg of 40- and 70,000 dalton dextrans and 12 mg of 110- and 150,000 dalton dextrans. Blood samples (10 μl) were taken at intervals over a 72 hr period, diluted to 50 μl with 0.9 NaCl and assayed for enzyme activity and radioactivity. Samples containing ^3H-dextrans were counted as previously described.

RESULTS AND DISCUSSION

Preparation of dextran-carboxypeptidase G_2 conjugates

The procedures and conditions adopted for the activation of dextran were those found by experimentation to give the highest degree of activation without precipitation of the dextran occuring through cross-linkage. In all cases the reaction product, following protein and glycine addition, was completely soluble, and Fig. 1 illustrates a typical elution profile of a dextran-CPG_2 reaction mixture separated on the Sephadex G150 column. The conjugates, prepared from dextrans of molecular weight 40,000 to 150,000 were found to elute sharply at the void volume (380 ml) in all cases, whereas free enzyme (650 ml) and the native dextrans (650–750 ml) were eluted much later. The void volume also contained soluble dextran polymers produced by intermolecular cross linkage not involving interaction with protein.

The average loss of enzyme catalytic activity was 30% following the overnight incubation step of the coupling reaction; no further activity was lost upon the addition of glycine. When fractions, corresponding to elution volumes between 380 ml and 450 ml, from the Sephadex G150 column were pooled, 50–60% of the enzyme activity loaded was recovered. No free enzyme activity was recovered from the column. The dextran and protein contents of the enzyme conjugates and the activity of radiolabelled dextrans used as controls, are listed in Table 1.

Table 1. Composition of the dextran–CPG_2 and ^3H–dextran preparations used for animal experiments

	Dextran (mg/ml)	Protein (μg/ml)	Enzyme activity (U/ml)	Specific activity (U/mg protein)	Radioactivity ^3H (μCi/ml)
Dextran 40–CPG_2	1.90	204.0	55	270	—
Dextran 70–CPG_2	3.48	180.8	22	122	—
Dextran 110–CPG_2	1.71	115.0	34	296	—
Dextran 150–CPG_2	1.58	112.5	29	257	—
^3H–dextran 40	20.0	—	—	—	1.14
^3H–dextran 70	20.0	—	—	—	0.75
^3H–dextran 110	12.0	—	—	—	0.50
^3H–dextran 150	12.0	—	—	—	0.57

^3H-labelled dextrans were prepared by reaction of cyanogen bromide-activated dextrans with ^3H-glycine.

Table 2. Estimated molecular weights of dextran–CPG_2 conjugates determined by gel filtration on Sephacryl S400 and Ultrogel AcA22

Conjugate	Molecular weight × 10^5 daltons* Sephacryl S400 D	Sephacryl S400 P	Ultrogel AcA22 D	Ultrogel AcA22 P
Dextran 40–CPG_2	5.1	6.3	6.0	6.9
Dextran 70–CPG_2	6.3	7.6	7.4	8.3
Dextran 110–CPG_2	5.5	7.1	9.1	10.0
Dextran 150–CPG_2	7.8	9.2	12.0	12.7

* Molecular weight of conjugates is shown based on both dextran (D) and protein (P) standards for comparison.

Estimation of molecular weights of dextran–CPG_2 conjugates by gel filtration

The molecular weights of the dextran–CPG_2 conjugates estimated by interpolation of calibration curves of dextran and protein standards chromatographed on Sephacryl S400 and Ultrogel AcA22 are presented in Table 2. Anomalies in the chromatographic behaviour of dextrans in gel filtration have previously been described and attributed to the random coil structure of the molecule [24, 25], hence for this study both dextran and protein molecular weight calibration markers were employed since it was predicted that the conjugate would possess dextran-like properties. In practice it was found that the molecular weights estimated from the dextran calibration curves were slightly lower than those estimated from the protein calibration curves, though the differences were not great. The molecular weights estimated by chromatography on the Sephacryl S400 column were in general lower than the corresponding results obtained using Ultrogel AcA22.

From the measured molecular weights it was possible to estimate the number of dextran molecules bound to a single CPG_2 molecule. In preparing these estimates (Table 3), it was assumed that dextran was present in sufficiently great excess (>20 × in reaction mixture) to ensure that conjugate contained only a single CPG_2 molecule. The results show that, as expected, the number of dextran molecules bound to CPG_2 decreases as the molecular weight of the dextran increases. These results mirror those reported for dextran-asparaginase conjugates [26]. A decrease in the number of dextran molecules bound with increasing molecular weight was predicted due to increasing steric hindrance between the larger molecules with masking of some of the available sites for binding on the enzyme surface.

Estimation of the degree of binding of dextran to CPG_2 by competitive binding with glycine

There are a large number of potential binding sites available on the CPG_2 molecule that can be occupied by dextran if each dextran is attached only by a single bond. The purpose of this experiment was to attempt to determine whether the dextrans are attached at multiple sites. This could not be obtained by conventional fluorimetric or colorimetric techniques because the carbamate groups generated by the cyanogen bromide activation are to some extent reactive and are present in sufficient quantity to mask the reaction of lysine residues on the protein.

The differences in glycine binding capacities between identical dextran samples when CPG_2 is either absent or present are presented in Table 4a, and show that the decrease in binding capacity is far greater than can be accounted for by the number of binding sites available on CPG_2, which possesses 31 lysine residues per sub-unit [27]. It appears likely, therefore, that in acting as a nucleus for dextran attachment, the CPG_2 molecule holds the dextran molecules in a sterically favourable position for them to form intermolecular bonds. Visual evidence for this hypothesis was provided by the observation that in these reaction mixtures, where CPG_2 concentration was high, a slight haziness developed compared to control samples.

This hypothesis was tested by repeating the experiment using an equivalent concentration of free amino groups to that present in CPG_2, in the form of 1,6-diaminohexane. This molecule would not be expected to react with dextran to form a similar nucleated structure to CPG_2 and would thus not reproduce the conditions favourable to the formation of intermolecular bonds. The results of this experiment are presented in Table 4b, and show that the decrease in glycine binding capacity corresponded

Table 3. Estimated numbers of dextran molecules per molecule of CPG_2 calculated from data presented in Table 2

	No. of dextran molecules per CPG_2 molecule Sephacryl S400	Ultrogel AcA22
Dextran 40–CPG_2	12	14
Dextran 70–CpG_2	9	10
Dextran 110–CPG_2	5	7
Dextran 150–CPG_2	5	7

Table 4. Estimation of the degree of reaction with (a) CPG_2 (5.3×10^{-9} mol) or (b) 1,6-diaminohexane (1,6-dah) (1.72×10^{-7} mol) by competitive binding with glycine

		Dextran 40		Dextran 70		Dextran 110		Dextran 150	
(a)	Time (hr)	Control	+CPG_2	Control	+CPG_2	Control	+CPG_2	Control	+CPG_2
No. of mol glycine binding per mol dextran	0	70.2	—	108.3	—	160.3	—	225.9	—
	24	36.6	19.0	58.5	35.7	80.1	46.0	123.3	69.0
Total amount of glycine displaced by CPG_2 (mol)	24	—	4.9×10^{-6}	—	3.7×10^{-6}	—	3.6×10^{-6}	—	4.2×10^{-6}
Ratio of glycine displaced by free-amino gps on CPG_2	24	—	14.8	—	11.2	—	10.9	—	12.7
(b)	Time (hr)	Control	+1,6-dah	Control	+1,6-dah	Control	+1,6-dah	Control	+1,6-dah
No. of mol glycine binding per mol dextran	0	57.6	—	111.9	—	152.7	—	188.3	—
	24	32.4	32.1	59.3	55.6	87.2	85.5	94.8	68.9
Total amount of glycine displaced by 1,6-dah (mol)	24	—	8.7×10^{-8}	—	5.9×10^{-7}	—	1.9×10^{-7}	—	1.9×10^{-6}
Ratio of glycine displaced by 1,6-dah	24	—	0.3	—	1.7	—	0.6	—	5.8

The total number of mol glycine displaced was calculated as the number of mol glycine displaced per mol of dextran multiplied by the number of mol dextran present. The ratio of glycine displacement by free-amino groups on CPG_2 was calculated as the total number of mol glycine displaced divided by the number of free-amino groups added as either CPG_2 (3.29×10^{-7} mol) or 1,6-diaminohexane (3.44×10^{-7} mol).

closely to the figure predicted for the amount of 1,6-diaminohexane added. In this case, formation of intermolecular bonds only occurred with dextran 150 and, even then, at a reduced level compared to the dextran CPG_2 conjugates.

A point of interest apparent in both experiments is the decay in glycine binding capacity noted even in the CPG_2-free control samples over the course of the experiment. This may be due to the chemical decay of the active sites generated or may be due to the formation of inter- or intramolecular bonds, or a combination of these factors. An indication that the latter process occurs to some degree at least is provided by the observation that little or no low molecular weight dextran remains in the dextran–CPG_2 preparation.

Determination of the resistance of CPG_2 and dextran–CPG_2 conjugates to proteolysis

A number of reports have described the enhanced stability of dextran–enzyme conjugates to proteolysis [17, 26]. Figures 2a and 2b show that CPG_2 was rapidly inactivated at 37° by trypsin and chymotrypsin respectively. In contrast the dextran 70– and dextran 150–CPG_2 conjugates retained their activity without loss even after 18 hr incubation (data not shown). Such a result might be expected in the case of trypsin, whose substrate amino acids are lysine and arginine, but the reason for the stability of these conjugates to chymotrypsin, whose substrates are the aromatic amino acids or those possessing bulky side chains is less clear. It has been suggested [26] that the dextran may form a steric barrier to the proteolytic enzyme, but data from this laboratory (Melton *et al.*, unpublished work) provides evidence that the far larger rabbit anti-CPG_2 antibody can bind and inactivate conjugated enzyme, suggesting that steric effects are unlikely to be the primary mechanism by which protection is afforded to dextran-linked CPG_2 from proteolytic enzymes.

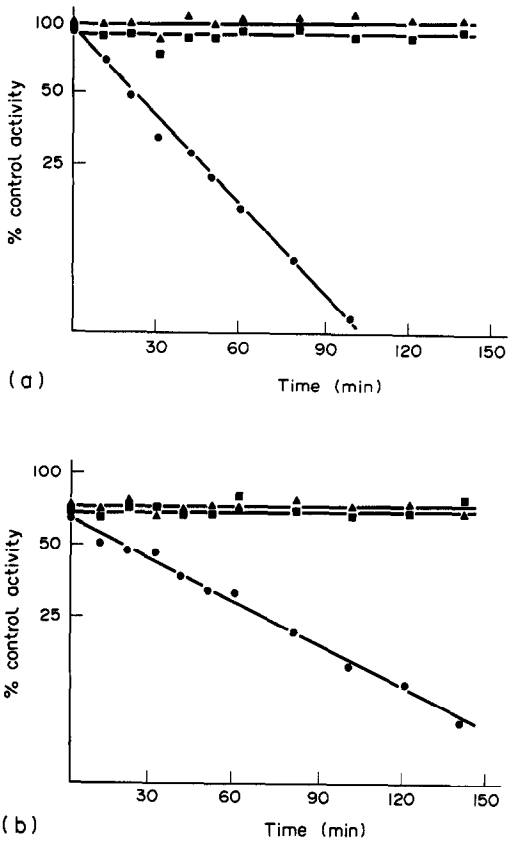

Fig. 2. Proteolytic inactivation of enzymic activity of CPG_2 (●), dextran 70–CPG_2 (■) and dextran 150–CPG_2 (▲) by (a) α-chymotrypsin and (b) trypsin. 100 μg of protein was reacted with either 10 μg of chymotrypsin of 5 μg of trypsin in 50 mM ammonium bicarbonate at 37°. Dextran 70 (10 mg) was added to the unmodified CPG_2 sample to compensate for the possible presence of unbound dextran in the dextran–CPG_2 conjugate samples.

The effect of pH on the stability of dextran–CPG_2 conjugates

CPG_2 shows enzymic activity over a broad pH range, with an optimum between 7.0 and 7.5 [20]. Coupling of the enzyme to dextran had no effect on either the range at which activity is present or the optimum activity range.

Kinetic properties of dextran–CPG_2 conjugates

Double reciprocal plots of reaction velocity (v) versus substrate concentration (S) followed Michaelis–Menten kinetics. From these data the K_m values of the dextran–CPG_2 conjugates for methotrexate were derived. Conjugates of dextran 150 and dextran 110 with CPG_2 showed slightly reduced K_ms of 0.9×10^{-5} M and 1.2×10^{-5} M respectively when compared to CPG_2 (0.8×10^{-5} M); however, dextran 70–CPG_2 and dextran 40–CPG_2 showed further reduction in affinity for substrate with values of 1.6×10^{-5} M and 2.6×10^{-5} M respectively. These results suggest that with greater numbers of lower molecular weight dextran molecules bound to CPG_2 some inhibition of substrate binding occurs and may provide further indirect evidence that the number of potential binding sites occupied is related to the number of dextran molecules present and not due to multiple site binding of dextrans.

Isoelectric point of dextran–CPG_2 conjugates

The isoelectric point of the dextran–CPG_2 conjugates, determined by agarose gel isoelectric focusing, were found to vary considerably from that of the native enzyme, which has a pI of 7.8 [20]. The pIs determined by this technique decreased from 4.75 for dextran 150–CPG_2 through 4.55 and 4.20 for dextran 110– and dextran 70–CPG_2 respectively, to 4.10 for dextran 40–CPG_2.

Plasma persistence of conjugates

Plasma persistence was expressed as the half-life ($T_{\frac{1}{2}}$) of native enzyme, dextrans and dextran–enzyme conjugates in normal and tumour-bearing mice (Table 5). Clearance of both CPG_2 and the dextran–CPG_2 conjugates followed first order kinetics throughout, with no evidence of an initial tissue distribution phase. Extrapolation of the clearance curves to determine the distribution volumes of the conjugates gave similar values in all cases, in the

Table 5. Persistence of carboxypeptidase G_2, dextrans and dextran-CPG_2 conjugates in normal and tumour-bearing Balb/c mice

Sample	$t_{\frac{1}{2}}$ (hr) Normal mice	$t_{\frac{1}{2}}$ (hr) Tumour-bearing mice
Native CPG_2	3.1 ± 0.4	6.5 ± 0.9
Dextran 40–CPG_2	14.3 ± 1.5	16.5 ± 2.9
Dextran 70–CPG_2	16.3 ± 2.4	18.9 ± 4.7
Dextran 110–CPG_2	23.0 ± 2.1	30.5 ± 7.5
Dextran 150–CPG_2	45.6 ± 7.6	>90
^{3}H–dextran 40	15.3 ± 1.3	—
^{3}H–dextran 70	16.8 ± 1.2	—
^{3}H–dextran 110	17.5 ± 1.2	—
^{3}H–dextran 150	18.9 ± 1.4	—

Data shown are means from groups of four mice with standard deviations. Analysis of group mean values from normal and tumour-bearing mice by *t*-text yielded a significant difference ($P > 0.01$) in half-life between native enzyme and each of the dextran: enzyme conjugates. There was also a significant difference ($P > 0.05$) in half-life as the size of the dextran increased, with the exception of dextran 40 compound with dextran 70 conjugates.

range 3.1 ± 0.4 ml/kg, corresponding to approximately 15% of body weight. The $T_{\frac{1}{2}}$ values of native CPG_2 and dextran 40–CPG_2 were very similar to those reported previously [6] and again demonstrated the marked increase in plasma $T_{\frac{1}{2}}$ (5-fold) when CPG_2 is coupled to soluble dextrans. Values for the ^{3}H-labelled dextrans showed very little difference in $T_{\frac{1}{2}}$ with molecular weight increase (40,000 to 150,000 daltons) and the measured values were all lower than figures commonly quoted for dextrans, namely 20–50 hr [18, 19]. This could be due to the cyanogen bromide activation of the dextrans necessary to introduce the tritiated glycine label into the molecule. In this respect they more closely resemble the dextran–CPG_2 conjugates used in the experiments and this was borne out in the similarity of $T_{\frac{1}{2}}$ values of ^{3}H-labelled dextran 40, 70 and 110 (15–18 hr) and the equivalent dextran–CPG_2 conjugates (14–23 hr) in normal mice. Only the dextran 150–CPG_2 conjugate showed a marked difference in $T_{\frac{1}{2}}$ (46 hr) compared to ^{3}H-labelled dextran 150 (19 hr). The extended $T_{\frac{1}{2}}$ (46 hr) of dextran 150–CPG_2 is more akin to the very high $T_{\frac{1}{2}}$ values described for dextran–asparaginase conjugates, prepared using a higher molecular weight range of dextrans from 110,000 to 2.0×10^6 daltons [8].

In tumour-bearing mice, the $T_{\frac{1}{2}}$ of native CPG_2 was increased from 3 hr to 6.5 hr. This effect has been described previously for CPG_2 [6] and is reminiscent of the lactate dehydrogenase virus associated effects reported for some other microbial enzymes of chemotherapeutic value [28, 29]. Linkage of CPG_2 to dextran 40 and 70 eliminates the difference in plasma persistence in normal and tumour-bearing mice, but with the higher molecular weight dextrans, particularly dextran 150, a significant difference in $T_{\frac{1}{2}}$ again appears.

The mechanism for clearance of dextran–CPG_2 conjugates has been studied and is discussed fully in a following paper [30]. It is highly unlikely that renal excretion of conjugate or native enzyme occurs as a primary route. Native dextrans are known to be taken up by the reticuloendothelial system [31]. Clearance may also be affected by prior interaction with plasma proteins. It has been shown that L-asparaginase can interact with α_2-macroglobulin *in vitro* [32], and it may be that similar interactions with plasma proteins *in vivo* are important in stimulating clearance. Dextran–CPG_2 conjugates prepared from dextrans of molecular weight 40,000 to 110,000 appear to be cleared on the basis of the dextran moeity alone. The much increased $T_{\frac{1}{2}}$ exhibited by the dextran 150–CPG_2 conjugate indicates a fundamental change in the clearance process, either through reduced recognition or through some physical size restriction.

We have demonstrated that a wide range of CPG_2 half-lives can be obtained by coupling to dextrans to varying molecular weight. This may be exploited to improve the therapeutic use of CPG_2 or other enzymes and proteins. Less frequent doses will be required through improved $T_{\frac{1}{2}}$ and different $T_{\frac{1}{2}}$ values may give a greater degree of dose control. It has also been shown previously that dextran-protein conjugates are less immunogenic than the corresponding native protein [13, 14] and this is currently under study with CPG_2 conjugates.

Although coupling CPG_2 to soluble dextrans may limit the ability of the enzyme to penetrate the capillary wall and deplete folate levels in the interstitial space of tissue, the much prolonged persistence in circulation would lead to a sustained depletion of total plasma folate without the requirement for repeated enzyme injection. This could be particularly significant in the current use of CPG_2 for the removal of circulating methotrexate in cases where impaired excretion of the drug leads to chronic toxicity.

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