Altered biodistribution of an antibody–enzyme conjugate modified with polyethylene glycol

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Summary Polyethylene glycol modification of the antibody–enzyme conjugate, F(ab')2-ASB7-CPG2, extends its duration in the circulation of nude mice bearing human colonic cancer xenografts (LSI74T). Increased concentration of modified conjugate is achieved in the tumour, but residual non-specific enzyme concentrations in normal tissue and blood demonstrate the fundamental requirement to remove or inactivate non-specifically held enzyme in this system.

Keywords: Antibody–enzyme; conjugate; polyethylene glycol

Antibody-directed enzyme prodrug therapy (ADEPT) holds promise for more selective delivery of cytotoxic drugs to tumours in vivo than can be expected from direct parenteral administration of the active drugs (Bagshawe et al., 1994). Critical factors for success are the differential concentration of enzyme at the tumour, rapid removal of unwanted enzyme from circulation and normal tissues to allow early administration and increased dose of the prodrug, and abrogation of the immunogenicity of the xenogenic proteins. Modifying proteins with polyethylene glycol (PEG) have been reported to prolong their circulatory half-lives in vivo (Chen et al., 1981; Katte et al., 1987; Berger and Pizzo, 1988) and lessen their immunogenicity (Abuchowski et al., 1977; Nucci et al., 1991). It is therefore of interest to determine whether this modification improves or reduces selective retention of the antibody–enzyme conjugate in a xenograft model.

In this study we report the linkage of PEG (5000 Da) to a conjugate comprising carboxypeptidase G2 and the F(ab')2 fragment of an anti-CEA monoclonal antibody (ASB7) and describe the altered distributions encountered in vivo. The localisation of F(ab')2-ASB7-CPG2 in nude mice bearing human colonic cancer xenografts has been well characterised (Sharma et al., 1991), making it a suitable model for studying the effects of modification with PEG. In addition, Sharma et al. (1994) have demonstrated that administration of galactosylated anti-carboxypeptidase monoclonal antibody (SB43gal) accelerates the clearance of F(ab')2-ASB7-CPG2 from the blood of nude mice, resulting in increased tumour–organ ratios without affecting conjugate localisation at the tumour. As a recent paper (Pedley et al., 1994) has compared in detail the distribution of pegylated antibody and antibody fragments in LS174T colonic cancer xenograft models in the context of radiolabelled antibody therapy, we concentrate in this report on the effect of pegylation on the requisite targeting of the enzyme to tumours and the effect of SB43gal on the biodistribution of the pegylated conjugate.

Materials and methods

Covalent attachment of PEG to F(ab')2-ASB7-CPG2

F(ab')2-ASB7-CPG2, prepared as reported (Melton et al., 1993), was kindly provided by Dr RG Melton, Division of Biotechnology, Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK. An aliquot of 120 mg of methoxy polyethylene glycol p-nitrophenyl carbamate (Sigma), was added to 10.5 mg of F(ab')2-ASB7-CPG2 in 3.2 ml of 0.2 M sodium phosphate buffer pH 7.2 (Veronese et al., 1985). After 1 h at room temperature unreacted PEG was removed by five successive ultrafiltration steps in an ultrafiltration cell (Amicon), using an XM-50 membrane. Each filtration was accomplished using 0.1 M sodium phosphate buffer, pH 7.2, as the dialysing solution. Pegylated protein was separated from unmodified protein by FPLC gel filtration using a Superose 5/12 HR column (Pharmacia) equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, and eluted with the same buffer.

Characterisation of PEG-F(ab')2-ASB7-CPG2

Protein content was determined by the bicinchoninic acid assay (Smith et al., 1985), which is unaffected by the presence of PEG. The amount of PEG attached to F(ab')2-ASB7-CPG2 was determined indirectly by quantitating the residual number of unmodified amino groups in the derivatised protein, using trinitrobenzene sulphonic acid according to the method of Habeeb (1966).

Enzyme assay

CPG2 activity was measured spectrophotometrically in 0.1 M Tris-HCl buffer, pH 7.3 (final volume 1.0 ml), containing 0.1 mM zinc chloride and 0.06 mM methotrexate (Sigma). The reaction was initiated by the addition of 10 μl of protein and enzyme activity was measured by the decrease in absorbance at 320 nm. One unit of CPG2 activity corresponds to 1 μmol of methotrexate hydrolysed min−1 (Sherwood et al., 1985).

Radiolabelling

Aliquots of 1 mg of F(ab')2-ASB7-CPG2 and 1 mg of PEG-F(ab')2-ASB7-CPG2 were each labelled with 1 mCi of 125I (Amersham) by the Iodogen method (Fraker and Speck, 1978). The specific activities of the 125I-labelled products were 0.65 μCi μg−1 for F(ab')2-ASB7-CPG2 and 0.54 μCi μg−1 for PEG-F(ab')2-ASB7-CPG2. Immunoreactivity of labelled product was evaluated by a carcinoembryonic antigen (CEA) binding assay. Briefly, 8 ng of each labelled product in 100 μl of phosphate-buffered saline (PBS) was added in sextuplicate to 96-well vinyl Costar plates previously coated with CEA (Wagener et al., 1983). After an incubation of 1 h at 37°C, the wells were washed five times with PBS, and the activity associated with the wells was counted in a gamma counter (LKB Wallac). The percentage of radioactivity associated with CEA was 59.0 ± 4.3% for 125I-Peg-F(ab')2-ASB7-CPG2 and 67.2 ± 2.8% for 125I-F(ab')2-ASB7-CPG2. Less than 6% of radioactivity was associated with non-specific binding for both proteins.
Biodistribution studies

Inbred MF-1 female athymic nude (nu/nu) mice 2–3 months old were used for all in vivo experiments. For the biodistribution experiment mice were implanted in the left flank with c.1 mm³ fragments of the human colon adenocarcinoma LS174T, and experiments were initiated 2–3 weeks after implantation when tumours had grown to approximately 1 cm in diameter. Mice were injected intravenously with 20 μg of 125I-F(ab')2-A5B7-CPG2, (specific activity 0.65 μCi μg⁻¹) or 20 μg of 125I-PFG-F(ab')2-A5B7-CPG2 (specific activity 0.54 μCi μg⁻¹). At 1, 6, 24, 48, 72 and 168 h after injection, groups of four mice were sacrificed and their liver, blood, kidney, lung, spleen, colon and tumour were excised and weighed. The tissues were dissolved in 7 M potassium hydroxide and radioactivity was counted in a gamma counter (LKB Wallac). Counts were corrected for radioactive decay and results were expressed as the percentage injected dose of radioactivity per gram of tissue (%ID g⁻¹). Statistical analysis was performed using a Student’s t-test for unpaired data.

The effect of SB434gal on the biodistribution of 125I-PEG-F(ab')2-A5B7-CPG2

The preparation of SB434gal has been previously reported (Sharma et al., 1994). Two groups of four athymic nude mice bearing LS174T human colon carcinoma xenografts were injected intravenously with 20 μg of 125I-PEG-F(ab')2-A5B7-CPG2 at time 0. One group of mice was injected intravenously with SB434gal (50 μg per mouse) at 22 h after receiving conjugate. At 24 h all the mice were sacrificed, tissues excised and the percentage injected dose per gram of tissue (%ID g⁻¹) determined.

Results

Purification and characterisation of PEG-F(ab')2-A5B7-CPG2

Purification of PEG-F(ab')2-A5B7-CPG2 was accomplished by a single gel filtration step. The superimposed gel filtration profiles of purified PEG-F(ab')2-A5B7-CPG2 and the unmodified protein determined separately on a superose S/12 HR column are shown in Figure 1. The profile for F(ab')2-A5B7-CPG2 showed two unresolved components (peaks a and b) which eluted over a region encompassing the elution volume of globular proteins with molecular weights in the range 180–250 kDa. From the molecular weights, components a and b were estimated to consist of 1 antibody–2 enzyme conjugate and 1 antibody–1 enzyme conjugate respectively. The PEG-F(ab')2-A5B7-CPG2 showed displacement of both components towards higher molecular weights in the range 250–300 kDa, owing to an increase in molecular size as a result of coupling to PEG. Determination of free lysine in PEG-F(ab')2-A5B7-CPG2 showed an average of 23 modified amino groups out of the available 107. From the rate of methotrexate turnover, PEG-F(ab')2-A5B7-CPG2 was calculated to have retained 95.4% of its initial specific enzyme activity.

Pharmacokinetics

Non-linear regression analyses of the whole blood clearance of 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 in tumour-bearing mice are shown in Figure 2. 125I-PEG-F(ab')2-A5B7-CPG2 was removed from the blood much more slowly and to a lesser extent than 125I-F(ab')2-A5B7-CPG2. One h after injection 23.74 ± 1.42% of the injected dose per ml (ID ml⁻¹) of 125I-PEG-F(ab')2-A5B7-CPG2 remained in blood compared with 7.32 ± 0.35% ID ml⁻¹ of 125I-F(ab')2-A5B7-CPG2. By 24 h only 0.19 ± 0.06% ID ml⁻¹ of 125I-F(ab')2-A5B7-CPG2 remained in blood compared with 6.28 ± 0.37% ID ml⁻¹ of 125I-PEG-F(ab')2-A5B7-CPG2.

The percentage of ID ml⁻¹ remaining in blood 168 h after injection for 125I-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 was 0.013 ± 0.003 and 0.083 ± 0.038 respectively. Half-lives of the terminal elimination phase (t1/2) for both proteins were similar, 23.8 h and 25.8 h for 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 respectively, suggesting the same catabolic rate. However, 125I-F(ab')2-A5B7-CPG2 always had a significantly reduced blood level compared with 125I-PEG-F(ab')2-A5B7-CPG2 at all time points.

Biodistribution study

The biodistributions of 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 were elevated in tumour-bearing mice from 1 h to 168 h. The percentage of the injected dose per gram determined in the tumour and in other organs is shown in Table 1. 125I-PEG-F(ab')2-A5B7-CPG2 showed significantly higher tumour uptake than 125I-F(ab')2-A5B7-CPG2 at all time points. Peak accumulation of 125I-PEG-F(ab')2-A5B7-CPG2 in the tumour occurred at 24 h with 6.33 ± 0.06% ID g⁻¹ (mean ± s.d.), compared with 2.29 ± 0.27% ID g⁻¹ for 125I-F(ab')2-A5B7-CPG2. Normal organ distribution was also significantly higher for 125I-PEG-F(ab')2-A5B7-CPG2 than for 125I-F(ab')2-A5B7-CPG2. The %ID g⁻¹ in colon ranged between 2.13 ± 0.43 at 6 h to 0.011 ± 0.004 at 168 h for 125I-PEG-F(ab')2-A5B7-CPG2 and from 0.73 ± 0.22 to
The ratios remained 10.5-fold lower in mice administered with SB43gal than in the untreated group. The ratio in liver diminished from 7.66 ± 0.85% ID g⁻¹ at 1 h to 0.05 ± 0.01% ID g⁻¹ at 168 h for 125I-F(ab')2-A5B7-CPG2, and from 4.21 ± 0.24% ID g⁻¹ to 0.020 ± 0.003% ID g⁻¹ for 125I-F(ab')2-A5B7-CPG2. Differences in uptake in normal tissue could be accounted for by the differences in blood clearance. Both conjugates showed increased tumour/blood ratios over time (Table II). 125I-F(ab')2-A5B7-CPG2 had the better ratios, ranging from 2.4 to 24 h to 25 at 168 h, whereas values of 0.65 at 24 h to 9.3 at 168 h were observed for 125I-F(ab')2-A5B7-CPG2, indicating a greater specific accumulation at the tumour site of 125I-F(ab')2-A5B7-CPG2.

The effect of SB43gal

The effect of SB43gal on the biodistribution of 125I-F(ab')2-A5B7-CPG2 in nude mice bearing LS174T xenografts is shown in Table III. The %ID g⁻¹ of 125I-F(ab')2-A5B7-CPG2 in blood was 10.5-fold lower in mice administered with SB43gal than in the untreated group. The %ID g⁻¹ values in other normal tissues were also lower in the mice administered with SB43gal except colon. This contrasted with the tumour where administration of SB43gal had no effect on the localisation of 125I-F(ab')2-A5B7-CPG2. Tumour--organ ratios were higher in mice administered with SB43gal, except for liver, kidney and colon where the ratios remained the same. For lung and spleen, tumour--organ ratio increased 2-fold and for blood the ratio increased 7-fold in the treated mice.

Table I  Tissue localisation of 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 in athymic nude mice bearing LS174T human colon adenocarcinoma xenografts

| Time | Tissue | 125I-PEG-F(ab')2-A5B7-CPG2 | 125I-F(ab')2-A5B7-CPG2 | P | 125I-PEG-F(ab')2-A5B7-CPG2 | 125I-F(ab')2-A5B7-CPG2 | P |
|------|--------|-----------------------------|------------------------|---|-----------------------------|------------------------|---|
| 1 h  | Blood  | 37.04±2.21                  | 11.42±0.55             | <0.0001 | 23.6±3.60                  | 6.09±0.78              | <0.0001 |
|      | Liver  | 7.60±0.85                   | 4.21±0.24              | <0.001  | 4.28±0.61                  | 2.72±0.69              | <0.005  |
|      | Kidney | 9.16±0.81                   | 4.04±0.79              | <0.001  | 6.02±0.67                  | 2.49±0.47              | <0.001  |
|      | Lung   | 9.84±0.61                   | 5.42±0.83              | <0.01   | 7.18±0.21                  | 3.45±0.72              | <0.001  |
|      | Spleen | 5.67±0.52                   | 3.99±0.63              | <0.01   | 3.37±0.77                  | 1.88±0.33              | <0.05   |
|      | Colon  | 1.61±0.09                   | 0.08±0.04              | <0.0001 | 2.13±0.43                  | 0.73±0.22              | <0.005  |
|      | Tumour | 2.55±0.40                   | 1.83±0.41              | NS*     | 5.36±1.95                  | 1.93±0.67              | <0.05   |

Two groups of athymic nude mice bearing LS174T human colon adenocarcinoma xenografts were injected with 20 μg of 125I-PEG-F(ab')2-A5B7-CPG2 or 20 μg 125I-F(ab')2-A5B7-CPG2. At the indicated times four mice were sacrificed from each group, tissues excised and the percentage injected dose per gram (%ID g⁻¹) was calculated. P-value is the statistical significance of the difference observed between the biodistribution of 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 at the time points. NS*, no statistically significant difference. The statistical significance was evaluated using the two-tailed Student's t-test.

Table II  Tissue organ ratios of 125I-PEG-F(ab')2-A5B7-CPG2 and 125I-F(ab')2-A5B7-CPG2 in athymic nude mice bearing LS174T human colon adenocarcinoma xenografts

| Tissue | Organ Ratios of 125I-PEG-F(ab')2-A5B7-CPG2 | Organ Ratios of 125I-F(ab')2-A5B7-CPG2 | P |
|--------|-------------------------------------------|----------------------------------------|---|
|        | Blood                                    | 0.07±0.23                              | 0.65±1.23 | 1.40±0.71 | 9.31 |
|        | Liver                                    | 0.33±1.25                              | 3.54±5.26 | 6.31±2.42 | 24.2 |
|        | Kidney                                   | 0.28±0.89                              | 3.10±4.92 | 5.78±2.42 | 24.2 |
|        | Lung                                     | 0.26±0.75                              | 1.84±3.66 | 4.00±2.42 | 24.2 |
|        | Spleen                                   | 0.45±1.59                              | 4.01±7.27 | 7.23±2.42 | 24.2 |
|        | Colon                                    | 1.58±2.52                              | 9.31±18.96| 24.78±11.00 | 110.0 |

Tumour--organ ratios were determined by dividing the %ID g⁻¹ of tumour by the %ID g⁻¹ of organ.

Discussion

The biodistribution of a parenterally administered exogenous protein in nude mice bearing the LS174T human colon adenocarcinoma will be affected by its size, charge, rate of
Table III  Effect of SB43gal on the clearance and biodistribution of 125I-PEG-F(ab')2-A5B7-CPG2 in athymic nude mice bearing LS174T human colon xenografts.

| Tissue          | 125I-PEG-F(ab')2-A5B7-CPG2 %ID g−1 T/O | SB43gal %ID g−1 | 125I-PEG-F(ab')2-A5B7-CPG2 %ID g−1 T/O | P       |
|-----------------|--------------------------------------|-----------------|--------------------------------------|---------|
| Blood           | 0.85 ± 0.13                          | 10.5            | 8.99 ± 0.72                          | 0.7     | <0.0001  |
| Liver           | 1.28 ± 0.18                          | 4.8             | 1.81 ± 0.39                          | 3.9     | <0.0001  |
| Kidney          | 0.87 ± 0.20                          | 7.0             | 1.30 ± 0.11                          | 4.6     | <0.0001  |
| Lung            | 0.98 ± 0.36                          | 7.0             | 2.59 ± 0.56                          | 2.4     | <0.0001  |
| Spleen          | 0.58 ± 0.23                          | 6.2             | 1.38 ± 0.15                          | 4.6     | <0.0001  |
| Colon           | 0.46 ± 0.24                          | 13.5            | 0.42 ± 0.11                          | 15      | <0.05    |
| Tumour          | 6.10 ± 0.73                          | 6.32 ± 0.35     | 6.32 ± 0.35                          | NS†     |          |

Two groups of four athymic nude mice bearing LS174T human colon carcinoma xenografts were injected intravenously with 20 μg of 125I-PEG-F(ab')2-A5B7-CPG2 at time 0, and one group of mice was injected intravenously with SB43gal (50 μg per mouse) at 22 h after receiving conjugate. At 24 h all the mice were sacrificed from each group, tissues excised and the percentage injected dose per gram (%ID g−1) was calculated. Tumour – organ ratios (T/O) were determined by dividing the %ID g−1 of tumour by the %ID g−1 of organ. P-value is the statistical significance of the difference observed in the biodistribution of 125I-PEG-F(ab')2-A5B7-CPG2 with and without clearance with SB43gal. NS, no statistically significant difference. The statistical significance was evaluated using the two-tailed Student’s t-test.

extravasation, rate of drainage into the lymphatic system and any uptake via specific receptors. The covalent linkage of PEG-5000 to F(ab')2-A5B7-CPG2 increases its molecular size significantly from 180–250 kDa to an effective molecular size of 250–300 kDa. Modification of the conjugate is accompanied by a minor loss of enzymatic activity. At the early (1 h) time point, there is no evidence of exclusion of pegylated materials from normal tissues, arguing that the complexes are not in a sized range for which postulated differences in fenestration between capillaries in tumour or normal tissues would play a predominant part in the distribution (Sung et al., 1990).

Our study shows specific tumour localisation of PEG-F(ab')2-A5B7-CPG2. Tumour uptake peaked at 24 h (6.33 ± 0.61% ID g−1). The specificity of retention was further demonstrated by the increasing ratios of PEG-F(ab')2-A5B7-CPG2 in the tumour compared with normal tissues with time (Table II). Even though from 1 h to 72 h, tumour – organ ratios were higher with F(ab')2-A5B7-CPG2 than with pegylated material by 168 h the ratios were the same for both proteins in all organs. Blood levels were the exception. Throughout the experiment tumour – blood ratios were lower with PEG-F(ab')2-A5B7-CPG2 than with the native conjugate. However at all time points, absolute levels of localisation at the tumour were approximately 3-fold greater for the former.

Non-linear regression analyses of the whole blood clearance curves of PEG-F(ab')2-A5B7-CPG2 and F(ab')2-A5B7-CPG2 demonstrated the persistence of PEG-F(ab')2-A5B7-CPG2 in blood (Figure 1). F(ab')2-A5B7-CPG2 and PEG-F(ab')2-A5B7-CPG2 had similar elimination rates, 25.8 h and 23.8 h respectively. The major difference between the two blood clearance curves is the significantly lower levels of F(ab')2-A5B7-CPG2 in the blood compared with PEG-F(ab')2-A5B7-CPG2 at all time points. At first sight, it might seem that this reflects a faster and more extensive extravascular diffusion of F(ab')2-A5B7-CPG2, compared with PEG-F(ab')2-A5B7-CPG2, hence greater clearance rates, as expected from the biodistribution of both materials in all tissue (Table I), even at early time points, shows that there is substantially more pegylated material in the tissues. This suggests that the component of the conjugate that has changed is the uptake into parenchymal cells of the liver, driven by carboxypeptidase G2 recognition (Melton et al., 1987), producing rapid elimination of the labelled antibody, and hence. It is also possible that the initial rapid decline in blood levels of F(ab')2-A5B7-CPG2 may have resulted from the selective removal of a unique fraction of the native conjugate that could occur if the injected compound were heterogeneous. We have confirmed that chemical coupling of the two components produces a product heterogeneous with respect to size (1:1 antibody-enzyme; 2:1 antibody-enzyme) and charge (Melton et al., 1993). Pegylation may mask the inherent differences between clearance rates of these two types of molecule.

What can be clearly stated, is that the proportion of injected conjugate retained at the tumour has been augmented by pegylation. This may be due to the extended circulatory life, reduction of natural clearance or, possibly, changes improving convection in the tumour owing to enhanced hydrophilicity. The saturation of available antigen on the tumour site is unlikely to be a limiting factor in present models (Sung et al., 1990). Pegylation appears to have facilitated egress of the conjugate molecules from the circulation into both tumour and normal tissues, which argues a serum concentration-dependent mechanism or altered physical characteristics or both, but does not suggest a transfer particular to tumour-associated capillaries. By inspection (Table I) the rate of loss of pegylated conjugate from the tumour is less markedly affected by its ingress, if allowance is made for the increased peak concentration.

The first requirement of improved localisation of conjugate at the tumour has been met by pegylation, but at the expense of a greater unwanted concentration of enzyme in blood and other tissues. To reduce the levels of circulating conjugate, SB43gal was administered 22 h after injection of the pegylated conjugate, when maximal tumour localisation had occurred and sufficient time was allowed for SB43gal-PEG-F(ab')2-A5B7-CPG2 complexes to clear from the plasma via the carbohydrate specific receptors in the liver. Previous work (Sharma et al., 1994; Rogers et al., 1995) suggests that the initial peak of active enzyme in the liver owing to accelerated clearance from the circulation has declined by 1 h after SB43gal administration. The effect of SB43gal on the biodistribution of PEG-F(ab')2-A5B7-CPG2 is to accelerate clearance from the circulation thereby reducing levels of the conjugate in plasma and extracellular fluid. However, in the tumour the absolute degree of localisation was not affected by accelerated clearance, giving rise to a 7-fold increase in tumour/blood ratio. It is essential that blood enzyme activity is reduced after tumour localisation has occurred to avoid prodrug activation in plasma leading to toxic effects (Bates et al., 1994). Ideally, a balance has to be struck to remove the non-antigen-bound conjugate as rapidly and completely as possible from the host after maximal retention levels have been achieved at the tumour site. This approach has been modelled successfully for unmodified conjugate by subsequent administration of SB43gal (Sharma et al., 1994), and we have now demonstrated that when the same approach is applied to the pegylated conjugate, enhanced tumour retention owing to pegylation is sustained while unbound conjugate can still be cleared rapidly.

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