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# Tumour Necrosis Factor Increases Tumour Uptake of Co-administered Antibody–Carboxypeptidase $G_2$ Conjugate

**R.G. Melton, J.A. Rowland, G.A. Pietersz, R.F. Sherwood and I.F.C. McKenzie**

**Increased tumour uptake of antibodies and antibody–drug conjugates has been demonstrated following pretreatment of animals with recombinant human tumour necrosis factor-α (rTNF-α) and interleukin 2 immunoconjugates. The experiments reported here were performed to determine whether improved tumour localisation of antibody–carboxypeptidase $G_2$ conjugates could be achieved, with a view to applying this technology to antibody-directed enzyme–prodrug therapy (ADEPT). B6CF$_1$ mice bearing the Ly-2.1$^+$ murine thymoma E3 were simultaneously injected with 2.0 μg rTNF-α and 3.5 μg (74 kBq) $^{125}$I-labelled murine anti-Ly-2.1-CPG$_2$ conjugate. Mice in control groups received phosphate buffered saline in place of rTNF-α. The conjugate corresponded in molecular weight to a mixture of 1:1 and 2:1 (CPG$_2$:IgG) conjugate and retained its antigen binding specificity and enzymic activity *in vitro*. A significant increase in tumour uptake was observed 24 h after administration when rTNF-α-treated animals were compared to controls (28.1 ± 9.7% / g and 11.6 ± 2.3% / g, respectively). Other tissues, most notably gut, skin and kidney also showed an increased localisation of conjugate. By 48 h, analysis of tissue:blood ratios demonstrated that although tumour:blood ratios were significantly higher in rTNF-α-treated animals ($P < 0.05$), all the other tissue:blood ratios were not significantly different between the two groups.**
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## INTRODUCTION

ANTIBODY-DIRECTED enzyme–prodrug therapy (ADEPT) is a novel approach to cancer therapy in which antibodies directed against tumour-associated antigens are used as vectors for enzymes which are capable of converting inactive prodrugs to active drugs [1, 2]. Careful design of prodrug and selection of enzyme should make it possible to produce prodrugs which are not susceptible to activation by endogenous enzymes and are therefore non-toxic to the host, but which can be activated by enzyme, bound via antibody to the target tumour, to exert a

highly localised toxic effect. Early studies have used the enzyme carboxypeptidase $G_2$ ($CPG_2$), a bacterial enzyme isolated from *Pseudomonas* sp., which cleaves glutamate from folic acid and its analogues to give the corresponding benzoic acid compound, as an activating agent for glutamate-deactivated benzoic acid mustard prodrugs [1]. This system has now been tested in pilot clinical studies on patients with advanced colorectal cancer [3]. For this application $CPG_2$ has been coupled to $F(ab')_2$ fragment of A5B7, a murine monoclonal antibody against carcinoembryonic antigen. Other workers have described a diverse range of enzyme–prodrug systems, including alkaline phosphatase together with phosphorylated cytotoxics such as etoposide phosphate and mitomycin C phosphate [4] or phosphorylated *p*-hydroxyaniline mustard [5]; other systems include glucuronidyl *p*-hydroxyaniline mustard and glucuronidase [6], penicillin-V-amidase with doxorubicin phenoxyacetamide [7] and cephalosporin-vinca alkaloid compounds which are substrates for β-lactamases [8].

Such systems are attractive for a number of reasons. A single molecule of enzyme is capable of activating many prodrug molecules, providing an amplification effect, whilst the active drug can be made small and highly diffusible. The enzyme can thus function extracellularly, eliminating the need to achieve binding and internalisation at every target cell. Given the reported antigenic heterogeneity of many human tumours [9–11] this should be an important means of attacking cells not expressing antigen which would not be susceptible to conventional immunoconjugates or immunotoxins.

Despite these perceived advantages, however, it is important to maximise the levels of antibody:enzyme conjugate localising at the tumour site whilst minimising uptake and persistence in non-target tissue, including blood. Conjugate retained in the blood and tissues can exert a systemic toxic effect by activation of prodrug, and for this reason a method of selectively clearing unbound conjugate using a galactosylated anti-$CPG_2$ antibody has been described [12]. The level of galactosylation used results in clearance of the galactosylated antibody complex by hepatic galactose receptors sufficiently rapidly to prevent the second antibody from reaching the tumour site and stripping bound conjugate from the tumour [12].

Co-administration of the vasoactive agent recombinant tumour necrosis factor α (rTNF-α) with monoclonal antibody immunoconjugates has also been demonstrated by Smyth *et al.* [13] and Russell *et al.* [14] to increase tumour localisation of the immunoconjugates. Leberthon *et al.* [15] have demonstrated that a similar effect can be achieved using antibody–interleukin-2 conjugates. It is likely that the effect of rTNF-α or IL-2 may be partly due to an increase in local inflammatory response resulting in vasodilation and increased permeability of the tumour vasculature. Such an effect might be beneficial when contemplating targeting antibody–enzyme conjugates, owing to their relatively large size, since antibodies alone have been reported to show only limited diffusion through tumour tissue [16, 17]. In a subsequent study Pimm *et al.* were unable to demonstrate increased localisation of antibody co-administered with rTNF-α although there was a demonstrable decrease in tumour blood flow [18]. If the 2-fold increase in absolute levels of tumour uptake reported by Smyth *et al.* [13] for antibody–drug conjugates could be reproduced with antibody–enzyme conjugate, it is axiomatic that tumour:blood ratios would be increased to at least a similar degree. This system might then be used to complement the antibody based clearing system and further enhance tumour:blood ratios. In this paper we describe the effect of tumour necrosis factor on the tumour localisation of carboxypeptidase $G_2$ ($CPG_2$) coupled to anti-Ly-2.1 antibody in the $B6CF_1$ mouse/E3 tumour model.

## MATERIALS AND METHODS

Two murine monoclonal antibodies were used in these studies, anti-Ly-2.1 which reacts with Ly-2.1 specificity [19], and A3C6 (anti-TFR), reactive with the human transferrin receptor (TFR) [13]. The anti-Ly-2.1 and A3C6 antibodies were isolated for ascites fluid [13]. rTNF-α was the gift of Boehringer Ingleheim, Frankfurt, Germany and had a specific activity of $2.6 \times 10^7$ U/mg. The folate-degrading enzyme $CPG_2$ was produced by the Division of Biotechnology (PHLS-CAMR), Porton Down [20]. The E3 clonal variant of the murine thymoma ITT(1)75NS(1) was maintained *in vitro* in Dulbecco's modified Eagle's medium (DME), supplemented with 10% newborn calf serum (Flow Laboratories) 2 mmol/l glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 U/ml penicillin (Commonwealth Serum Laboratories) and 100 μg/ml streptomycin (Glaxo Laboratories). For *in vivo* experiments E3 was maintained by serial passage in the ascites form in $B6CF_1$ mice; cells from the ascites fluid were washed and centrifuged (400 *g*, 5 min) twice in DME and phosphate buffered saline (PBS) (pH 7.3), resuspended in PBS and injected subcutaneously into the abdominal wall of mice, and allowed to develop into palpable tumours before commencing treatment.

### *Preparation of antibody–enzyme conjugate*

Antibody–enzyme conjugate was prepared using the heterobifunctional agents succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) and 2-mercapto-[S-acetyl]acetic acid *N*-hydroxysuccinimide (SATA) [12]. The reaction conditions were based on the optimum figures used for the coupling of $CPG_2$ to the A5B7 monoclonal antibody: 5-fold molar excess of SMPB:$CPG_2$ and 4-fold molar excess of SATA:IgG [21]. Antibody–enzyme conjugate thus prepared was isolated from high molecular weight aggregates and uncoupled components by gel filtration chromatography using a calibrated column (16 × 700 mm) of Superdex G200. The column was equilibrated in PBS and eluted with the same buffer at a flow rate of 1.0 ml/min. Fractions containing material corresponding in molecular weight to 2:1 and 1:1 conjugate (316 kD and 233 kD respectively) were pooled and rechromatographed on the same column and samples from pooled fractions were run on 4–15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (Pharamacia Phastgels run for 60 μh) under non-reducing conditions. The gels were scanned on a densitometer (Joyce-Loebel Chromoscan 3) for estimation of relative amounts of conjugates and free antibody. Fractions containing predominantly 1:1 and 2:1 conjugate (enzyme:IgG) were pooled and concentrated to about 3 mg/ml total protein. Lactose (1% w/v) was added, the solution filter sterilised, dispensed in 1 ml aliquots, snap-frozen using a solid $CO_2$/acetone bath and lyophilised. The lyophilised conjugate was sealed under nitrogen and stored at 4°C until used for *in vitro* and *in vivo* studies.

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The protein content of lyophilised vials was determined by the Coomassie blue G250 dye binding assay [22], following reconstitution of a single vial in 1 ml distilled water. The enzyme activity of antibody-linked $CPG_2$ was measured spectrophotometrically at 37°C in Tris–HCl buffer with 0.06 mmol/l methotrexate as substrate in a total volume of 1 ml. The reaction was initiated by the addition of enzyme and followed by the decrease in absorbance at 320 nm [20].

*Radiolabelling of conjugate*

Anti-Ly-2.1–$CPG_2$ conjugate (100 μl, 2.5 mg/ml) was labelled using the chloramine T method [23]; 18.5 MBq of carrier-free $Na^{125}I$ (Amersham International Ltd), and 3 μl of chloramine-T (1 mg/ml) were mixed with the protein for 2 min at room temperature. The reaction was terminated by the addition of 3 μl of sodium metabisulfite (2.4 mg/ml). Iodinated antibody–enzyme conjugate was separated from free iodine by gel filtration using a Sephadex G25 column (PD10, Pharmacia).

*Antigen binding activity of conjugate*

The antigen binding of anti-Ly-2.1–$CPG_2$ conjugate was demonstrated by a fluorescent-label assay. E3 cells (0.5 ml, $10^5$ cells/ml) suspended in PBS containing 2% fetal calf serum and 0.025% sodium azide were incubated with dilutions of conjugate, native anti-Ly-2.1, or anti-transferrin receptor (negative control) antibodies, ranging from 100 μg/ml to 0.1 μg/ml. After incubation for 1 h at 4°C, the cells were washed three times with the same buffer, 100 μl of fluorescein isothyocyanate (FTIC)-labelled sheep anti-mouse antibody added (Silenius Laboratories, Victoria, Australia) and incubated for a further 1 h at 4°C. The cells were then washed a further three times with buffer and were suspended in 1 ml buffer. The presence of fluorescent label was determined using a Becton-Dickinson FACSCAN C fluorescence-activated cell sorter. The selectivity of binding of anti-Ly-2.1–$CPG_2$ conjugate to cells expressing the Ly-2.1 antigen was measured by radioimmunoassay in which radiolabelled conjugate was allowed to react with Ly-$2.1^+$ or Ly-$2.1^-$ cells. Thymus cells from CBA/1 (LY-$2.1^+$) or Balb/c (Ly-$2.1^-$) mice were suspended in 0.83% ammonium chloride solution for 5 min then collected by centrifugation (400 *g* for 5 min) and resuspended in 1% bovine serum albumin (BSA)/PBS at a concentration of $2.5 \times 10^7$/ml. The cells were plated out in duplicate rows in flexible polyvinyl chloride 96-well plates (Costar) and various dilutions of $^{125}I$-labelled antibody–enzyme conjugate added to give a range of radiolabel from 500 000 to 250 cpm per well. The antibody–enzyme conjugate was incubated with the cells for 1 h at 37°C after which the cells were collected by centrifugation of the plate and washed with 1% BSA/PBS three times. The plates were then cut up and radiolabel bound to cells determined by counting individual wells in a β-counter.

*Antibody–enzyme conjugate activity* in vitro

The presence of active enzyme in conjugate was demonstrated by means of cytotoxicity assay against methotrexate. E3 cells were suspended at $10^6$ cells/ml in DME containing 10% heat inactivated newborn calf serum, 2 mmol/l glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml. The cell suspension was divided into three aliquots of 4 ml and native $CPG_2$ (70 μg in 72 μl PBS), anti-Ly-2.1–$CPG_2$ (200 μg in 72 μl PBS) or 72 μl PBS was added and the mixtures incubated for 1 h at 37°C. The cells were then washed three times with DME and were plated out in polyvinyl chloride 96-well plastic plates (Dynatech Laboratories Inc, Alexandria, Virginia, U.S.A.) in duplicate columns. Various concentrations of methotrexate (Sigma) from $2.75 \times 10^{-5}$ mol/l to $1.35 \times 10^{-8}$ mol/l were added across the plate, and incubated for 16 h at 37°C; 50 μl of $^3H$-deoxyuridine (25 nmol, $2.2 \times 10^6$ cpm) (Amersham) were then added and incubation continued for a further 3 h. The cells were then harvested onto glass fibre filter discs which were dried for 10 min at 80°C and counted on a β-scintillation counter to measure cell uptake of $^3H$-deoxyuridine.

*Biodistribution studies*

Groups of four $B6CF_1$ mice, bearing palpable Ly-$2.1^+$ E3 thymoma tumours implanted subcutaneously in the abdominal wall, received intravenous injections of 3.5 μg anti-Ly-2.1-$CPG_2$ conjugate (74 kBq) mixed with 2 μg rTNF-α in a total volume of 200 μl PBS. Mice in control groups received the same dose of conjugate without TNF. Groups of animals were killed at 24 and 48 h for the determination of tissue uptake which was calculated as the mean percentage injected dose/g of tissue for a group of four mice.

## RESULTS

*Preparation of antibody–enzyme conjugate*

The isolation of crude anti Ly-2.1–$CPG_2$ conjugate from uncoupled anti-Ly-2.1 and $CPG_2$ is illustrated in Fig. 1a. Conjugate was well separated on a calibrated Superdex G200 column from uncoupled $CPG_2$ and, to a lesser extent, anti-Ly-2.1. The conjugate produced was a mixture of material corresponding to an estimated Mr 235 kD from the column calibration, and greater, tentatively identified as 2:1 and 1:1 ($CPG_2$:anti-Ly-2.1) together with larger conjugates. This necessitated a second purification step to further isolate 1:1 conjugate; the elution profile obtained for the rechromatographed material is illustrated in Fig. 1(b) and shows that the final pool was largely 1:1 conjugate with the peak again corresponding to Mr 235 kD. A 4–15% gradient SDS–PAGE gel run on samples from across the peak of the second pool under non-reducing conditions (Fig. 2) showed that the conjugate was contained in the fractions represented by track 4, which contained approximately 48% 1:1 conjugate plus 27% 2:1 conjugate and less than 20% free anti-Ly-2.1, as measured by a densitometric scan of the gel. The fractions corresponding to track 4 were pooled and concentrated for freeze-drying. These fractions contained a mixture of predominantly 1:1 enzyme–antibody conjugate ($M_r$ 193 kD for a conjugate containing one IgG molecule and one $CPG_2$ subunit) together with a lesser amount of 2:1 conjugate ($M_r$ 233 kD and a small proportion of free antibody($M_r$ 150 kD). $CPG_2$ is not stable in SDS–PAGE even under non-reducing conditions and is only ever seen as a band corresponding to the subunit molecular weight (43 kD); covalent linkage of $CPG_2$ to antibody does not stabilise the $CPG_2$ subunit structure, so that subunits not covalently attached to IgG are labile even if associated with a subunit which is covalently linked. It is thus not possible with this technique to determine whether non-coupled $CPG_2$ is present since 1:1 antibody conjugate will also give a band corresponding to free $CPG_2$ subunit, however $CPG_2$ is stable under the conditions used for gel filtration and the Superdex G200 column did show that the principle products had $M_r$ 235 kD and 316 kD, which correspond to that predicted for 1:1 and 2:1 conjugate, respectively. No material was eluted at the volume corresponding to the $CPG_2$ calibration peak. The specific activity of the enzyme component in the lyophilised product was determined as being 102 U/mg.

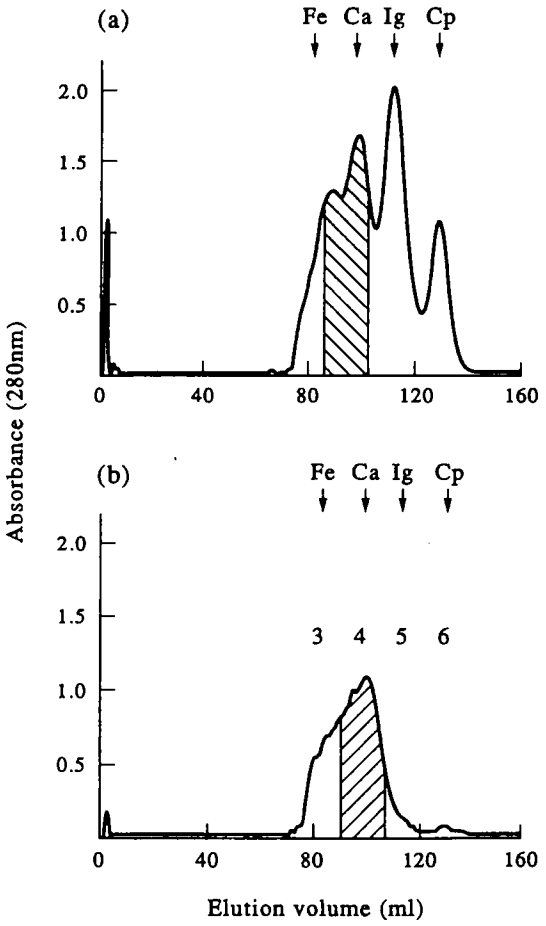


**Fig. 1. Purification of anti-Ly-2.1–$CPG_2$ conjugate. The elution profiles of (a) a crude conjugate preparation of anti-Ly-2.1–$CPG_2$ from a calibrated column (16 mm × 700 mm) of Superdex G200. The shaded fractions were pooled, concentrated and rechromatographed on the same column (b) using identical running conditions. Samples of the product were taken at the points indicated by the marked numbers for examination by SDS–PAGE, as shown in Fig. 2. The molecular weight calibration proteins used were Ferritin (Fe), 440 kD; catalase (Ca), 232 kD; mouse IgG (Ig), 150 kD; carboxypeptidase $G_2$ (Cp), 83 kD and the elution volume of the respective peaks are indicated by the labelled arrows.**

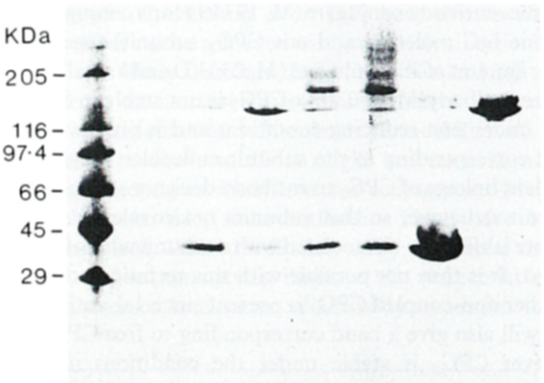


**Fig. 2. SDS–PAGE analysis of purified anti-Ly-2.1–$CPG_2$ conjugate. SDS–PAGE analysis of the anti-Ly-2.1–$CPG_2$ conjugate was performed using precast 4–15% gradient Phastgels run for 60 vh and stained with Phastgel blue R. Track 1: anti-Ly-2.1, track 2: $CPG_2$, tracks 3–6: marked fractions shown on Fig. 1b, track 8: high molecular weight standards (SDS-7, Sigma).**

*Determination of antigen binding activity of conjugate*

Matched amounts of anti-Ly-2.1 in the form of conjugate or native antibody were used. The antigen binding activity of the conjugate was determined by means of a fluorescence assay in which the specific antibodies and non-specific controls were titrated against a fixed number of cells, with bound antibody being measured by an FITC-labelled sheep-anti-mouse IgG antibody. In this assay the amount of antibody binding specifically to the target cells will increase as the total amount of specific antibody increases until all the available antigen is saturated. The results obtained are illustrated in Fig. 3 and show that the antigen binding activity of the conjugate was very similar to that of native antibody, although there was an indication that native anti-Ly 2.1 was saturating available sites whereas conjugate did not appear to do so; in contrast, there was no reaction between Ly-2.1$^+$ cells and the anti-TFR control, indicating that the binding observed with anti-Ly-2.1–$CPG_2$ was indeed owing to specific binding of the antibody to the cells, rather than non-specific binding. At antibody concentrations of 100 μg/ml the samples containing specific antibody showed 10-fold higher binding to the E3 cells than the non-specific control.

In addition to demonstrating specificity of binding to Ly-2.1$^+$ cells in comparison with non-specific anti-transferrin receptor antibody, it was also demonstrated by means of a radioimmunoassay that the antibody–enzyme conjugate bound specifically to Ly-2.1$^+$ cells but not to Ly-2.1$^-$ cells. The results of this experiment are illustrated in Fig. 4 and show that as the radiolabelled conjugate was titrated against a fixed concentration of cells, and the amount of bound radiolabel plotted versus total activity added, the amount of conjugate binding to the Ly-2.1$^+$ cells increased, but the amount binding to Ly-2.1$^-$ cells did not, clearly demonstrating the specificity of binding to antigen-positive cells.

*Enzyme activity of conjugate*

A cytotoxicity assay was used to confirm that enzyme activity present in the conjugate preparation was covalently linked with

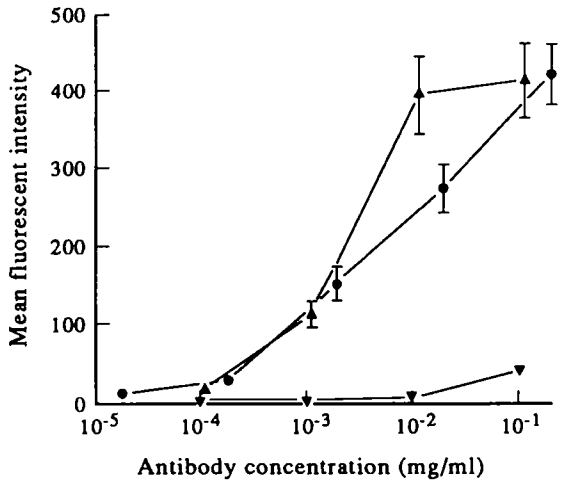


**Fig. 3. FACS analysis of antigen binding activity of anti-Ly-2.1–$CPG_2$ conjugate. E3 cells were incubated with various dilutions of anti-Ly-2.1–$CPG_2$ conjugate (●), native Ly-2.1 (▲) or anti-transferrin receptor (▼) (negative control) antibodies. After incubation for 1 h at 4°C the cells were washed, FITC-labelled sheep anti-mouse antibody added and incubated for a further 1 h at 4°C. The cells were then washed and subjected to FACS analysis. Results shown are means of duplicate determinations and error bars show the standard error of the mean.**

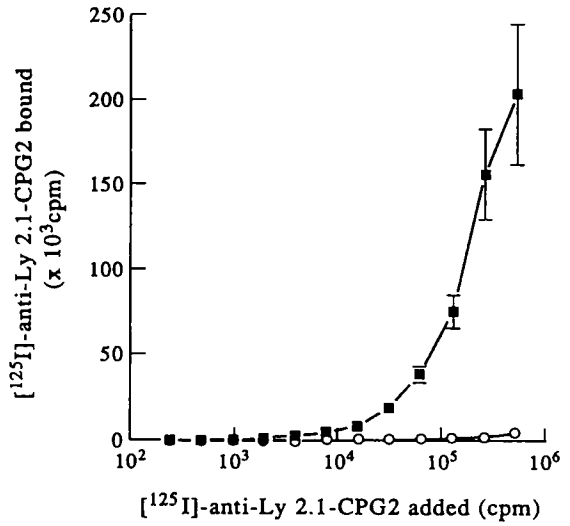


**Fig. 4. Specificity of anti-Ly–2.1 $CPG_2$ conjugate binding to Ly 2.1 antigen. Thymus cells from CBA/1 (■, Ly-2.1⁺) or Balb/c (○, Ly-2.1⁻) mice were plated out in duplicate in flexible 96-well plates and various dilutions of $^{125}$I-labelled antibody–enzyme conjugate added to give a range of radiolabel from 500 000 to 250 cpm per well. After 1 h incubation the cells were washed and individual wells of the plate counted in a τ-counter.**

the antibody moiety. $CPG_2$ cleaves the terminal glutamate from methotrexate to yield the non-toxic pteroate form. Incubation of Ly-2.1-positive cells with anti-Ly-2.1–$CPG_2$ conjugate would be expected to give protection against methotrexate toxicity owing to degradation of methotrexate by enzyme, after washing to remove unbound conjugate. Native $CPG_2$ would not be expected to bind to the cells and so should not protect against methotrexate toxicity after washing. The results of this experiment were calculated as percentage of [$^3$H]deoxyuridine uptake with respect to untreated control cells and are presented in Fig. 5, plotted against methotrexate concentration. The PBS control showed an $IC_{50}$ for methotrexate of $8.0 \times 10^{-7}$ mol/l, whereas

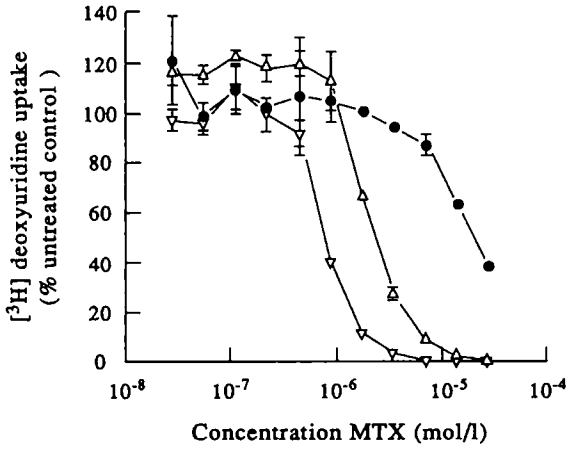


**Fig. 5. Retention of enzymic activity of anti-Ly 2.1–$CPG_2$ conjugate. A suspension of E3 cells was divided into three aliquots of 4 ml and incubated with native $CPG_2$ (μ) (70 μg in 72 μl PBS), anti-Ly-2.1–$CPG_2$ (●) (200 μg in 72 μl PBS) or 72 μl PBS (▽) for 1 h at 37°C. The cells were washed and plated out in 96-well plastic plates in duplicate columns with various concentrations of methotrexate from $2.75 \times 10^{-5}$mol/l to $1.35 \times 10^{-8}$mol/l. After incubation for 16 h viable cells were measured by a $^3$H deoxyuridine uptake assay. Results shown are means of two separate determinations. Error bars show the standard error of the mean.**

cells pretreated with anti-Ly-2.1–$CPG_2$ showed a 30-fold increase in $IC_{50}$ to $2.2 \times 10^{-5}$ mol/l demonstrating specific antibody-mediated binding of conjugate to the cells. When native $CPG_2$ was used there was a 2-fold increase in $IC_{50}$ to $1.5 \times 10^{-6}$ although this was largely due to the curve being shifted upwards, suggesting that $CPG_2$ or a constituent of its buffer may have been exerting a mitogenic effect. Such an effect has not previously been observed with $CPG_2$ (R.B. Pedley, personal communication), suggesting that this may have been artefactual.

*Influence of rTNF-α on biodistribution of anti-Ly-2.1-$CPG_2$*

Four groups of mice were used to provide samples at 24 h (groups A and B) and 48 h (groups C and D) after intravenous injection via the tail vein. For each time point there was a control group (groups B and D at 24 h and 48 h, respectively) which received only anti-Ly-2.1–$CPG_2$ conjugate together with a group which received conjugate plus 2 μg rTNF-α (groups A and C) administered simultaneously as a mixture.

The effect of rTNF-α treatment on the biodistribution of anti-Ly-2.1–$CPG_2$ conjugate is illustrated in Fig. 6. A significant increase in tumour uptake of conjugate was observed 24 h after co-administration of conjugate and rTNF-α, when TNF-treated animals (group A) were compared to controls (group B) (28.1 ± 9.7%/g and 11.6 ± 2.3%/g, respectively). Other tissues, most notably gut, skin and kidney, also exhibited transient increased localisation of conjugate, but by 48 h these effects had declined so that whilst TNF-treated animals (group C) displayed 2-fold greater tumour uptake than untreated controls (group D) there was no significant difference in the conjugate uptake in other tissues. When tissue to blood ratios were calculated these differences were seen to be more pronounced. The tissue to blood ratios for all groups are presented in Table 1. At 24 h, TNF-treated animals (group A) had a tumour to blood ratio of 1.88, compared with 0.76 for controls (group B). For the TNF-treated animals (group A) the tumour to blood ratio was approximately 1.5–10-fold higher than other tissues and the untreated controls (group B) showed ratios which were comparable. By 48 h the ratios had increased to a value of 10–20-fold for TNF-treated animals (group C) but remained in the region of 2–10-fold for untreated controls. There was a consistent, but not statistically significant, decrease in the amount of conjugate in blood in TNF-treated animals in addition to the increased tumour uptake noted. The statistical significance of the results

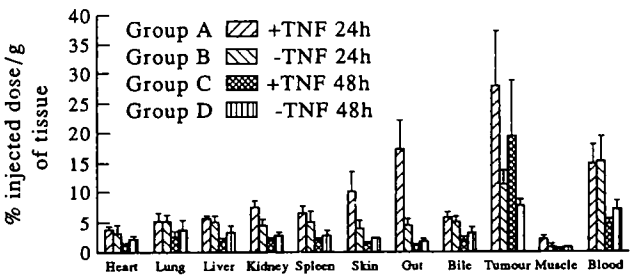


**Fig. 6. Tumour localisation of anti-Ly 2.1–$CPG_2$ conjugate. Groups of four $B6CF_1$ mice bearing palpable Ly-2.1⁺ E3 thymoma tumours received intravenous injections of anti-Ly-2.1–$CPG_2$ conjugate as described in the text (groups A and C). Mice in control groups B and D received the same dose of conjugate without TNF. Groups of animals were killed at 24 h (groups A and B) and 48 h (groups C and D) for the determination of tissue uptake, which was calculated as the mean percentage injected dose per gram of tissue for a group of four mice. Error bars show the standard error of the mean.**

*Table 1. Tissue:blood ratios*

| Group | A +TNF, 24h | B −TNF, 24h | C +TNF, 48h | D −TNF, 48h |
|---|---|---|---|---|
| Heart | 0.25±0.08 | 0.20±0.05 | 0.29±0.10 | 0.28±0.06 |
| Lung | 0.39±0.07 | 0.33±0.04 | 0.60±0.32 | 0.49±0.17 |
| Liver | 0.37±0.10 | 0.37±0.03 | 0.45±0.18 | 0.44±0.09 |
| Kidney | 0.51±0.14 | 0.30±0.06 | 0.43±0.17 | 0.39±0.07 |
| Spleen | 0.45±0.12 | 0.33±0.05 | 0.45±0.14 | 0.38±0.08 |
| Skin | 0.69±0.26 | 0.26±0.04 | 0.36±0.07 | 0.32±0.08 |
| Gut | 1.16±0.37 | 0.30±0.05 | 0.25±0.05 | 0.27±0.10 |
| Bile | 0.40±0.12 | 0.34±0.03 | 0.48±0.21 | 0.44±0.09 |
| Tumour | 1.88±0.74 | 0.76±0.10 | 4.22±2.34 | 1.10±0.29 |
| Muscle | 0.16±0.03 | 0.07±0.04 | 0.18±0.10 | 0.14±0.06 |

Tissue to blood ratios were calculated from the biodistribution data presented in Fig. 6 and show means ± S.D. $(n-1)$ for groups of four mice.

shown in Table 1 was calculated by performing paired *t*-tests on the groups A and B, and groups C and D. These results are presented in Table 2. These results show that at 24 h there were significant differences in the tissue to blood ratios between TNF-treated animals and controls in a number of tissues, most notably skin, gut, muscle and tumour. By 48 h, however, the only tissue to be significantly different between TNF-treated and control groups was tumour.

## DISCUSSION

For successful elimination of tumour cells using immunoconjugates of conventional cytotoxic drugs or toxins it is essential that the conjugate binds to every target cell and consequently antigen-negative cells are not susceptible to such conjugates. The ADEPT system offers the potential of being able to overcome heterogeneity of antigen expression in tumours owing to active drug being generated at the active site, so that active drug is able to diffuse to adjacent antigen-negative cells—the so-called bystander effect. There is also some evidence that active drug thus generated in intimate contact with the target cells is more effective than a similar concentration present in the general environment [24]. Nevertheless, in order to achieve site-specific activation of prodrug it is necessary to have high levels of conjugate present in tumour with minimal residual activity in other tissues. Typically, however, tumour:normal tissue ratios for major organs such as liver, kidney, lung and spleen are less than 5:1 even at 72 h after administration, and in the case of blood a ratio of 0.5:1 would be more typical. Since there is a large quantity of normal tissue and a relatively small amount of tumour (< 15% total body weight as tumour) it follows that the total amount of conjugate present in normal tissues is likely to greatly exceed that localised in the tumour. The measured biodistribution may be distorted somewhat by the fact that the radiolabel used to measure tissue uptake may not remain associated with the conjugate, but it is nevertheless clear that for ADEPT to be successful it is necessary to maximise tumour uptake and minimise non-specific retention of conjugate. These issues have been partially addressed to some extent by the galactosylated clearing antibody system described by Sharma *et al.* [12], which provides a mechanism for clearance of unbound conjugate from the blood. Such a system only decreases non-specific retention, although it does result in enhanced tumour:tissue ratios for most tissues, the principal exception being the liver. Here the observed localisation is most likely to be owing to uptake of galactosylated antibody–antigen complex and it is thus highly unlikely that the conjugative will be active, since the SB43 antibody used is directed at the active site of the enzyme and inactivates it.

*Table 2. P values from t-test*

| | Organ:blood ratio 24 h | 48 h |
|---|---|---|
| Heart | 0.149 | 0.433 |
| Lung | 0.063 | 0.289 |
| Liver | 0.354 | 0.830 |
| Kidney | 0.028 | 0.491 |
| Spleen | 0.078 | 0.134 |
| Skin | 0.005 | 0.242 |
| Gut | 0.002 | 0.414 |
| Bile | 0.330 | 0.651 |
| Tumour | 0.022 | 0.030 |
| Muscle | 0.020 | 0.269 |

The statistical significance of the differences in tissue:blood ratios shown in Table 1 was calculated by means of paired *t*-test; the results show the significance of the difference in tissue:blood ratios between mice receiving rTNF-α plus conjugate and control animals which received conjugate alone at the two time points shown.

An alternative approach is to try to achieve enhanced tumour uptake of conjugate as described by Smyth *et al.* [13] and Russell *et al.* [14] using rTNF-α; increased tumour uptake should result in lower amounts in other tissues. The experiments reported in this paper describe the effect of TNF on the localisation of an antibody–enzyme conjugate constructed using one of the most promising candidate enzymes for use in ADEPT systems; the conjugate was shown to be able to specifically bind to cells bearing the Ly-2.1 antigen but not cells which were Ly-2.1-negative. The demonstration of a specific protective effect against methotrexate toxicity owing to bound conjugate is particulary encouraging in suggesting that significant amounts of conjugate can bind to cells, sufficient to give a pronounced biological effect. This assay closely mimics the ADEPT application albeit reversing the function of $CPG_2$ to produce an innocuous compound from a potent cytotoxin. Although $CPG_2$-mediated cytotoxicity owing to prodrug activation has been demonstrated *in vitro* [1], the experiments reported to date have used native enzyme without antibody present and thus the results described here are the first in which conjugated $CPG_2$ has been shown to bind in sufficient quantities to produce a measurable biological effect, although other groups using alternative prodrug/enzyme systems have demonstrated such an effect [4–7].

The *in vivo* studies reported here demonstrate that a significant increase in tumour uptake can be achieved without a concomitant increase in non-target tissue. The level of localisation increased about 2-fold compared with controls which did not receive rTNF-α, whilst there was only a transient increase in localisation in other tissues in the TNF-treated animals, and this had receded by 48 h. The results reported here are thus similar to those reported by Smyth *et al.* [13] for *N*-acetyl melphalan–anti Ly-2.1 conjugate, although in the earlier study rTNF-α was administered intraperitoneally 24 h prior to intravenous injec-

tion of the conjugate. The timing of TNF and conjugate administration has been reported by Russell *et al.* [14] to be an important factor in studies using aminopterin–antibody conjugate, where it was found that an interval of 4 h between administration of rTNF-α and conjugate resulted in significantly lower tumour uptake of conjugate by comparison with animals which received TNF and conjugate simultaneously. The experiments described here were designed around the parameters described by Russell *et al.* [14] and appear to have produced very similar results, confirming the reproducibility of the model. The transient increase in gut uptake mirrored that found in stomach in the earlier experiments, although the increased kidney uptake did not. A study reported by Pimm *et al.* [18] in which the effect of rTNF-α on tumour localisation of monoclonal antibodies and tumour blood flow was measured, indicated that the increased localisation reported here for antibody–enzyme conjugate may not result from decreased tumour blood flow in either of the human tumour models studied. To some extent this supports the hypothesis of Russell *et al.* [14] that the increased tumour localisation may be owing to tumour-specific vascular damage induced by rTNF-α although it is also possible that the human tumour xenografts responded differently to the murine thymoma used in our studies. The rTNF-α system also gave an enhancement of tumour to normal tissue ratios which was comparable with the galactosylated SB43 clearing antibody system reported by Sharma *et al.* [12] who achieved a tumour:normal tissue ratio of about 2:1. In practice the latter value may be an underestimate since the SB43 antibody is directed at the active site of $CPG_2$ and so although radiolabel is present in the tissues it may be associated with SB43 inactivated enzyme. There is, therefore, justification for extending these studies to investigate their possible relevance to ADEPT. If antitumour trials prove successful in demonstrating an enhanced therapeutic effect using rTNF-α, a combination of this regime with subsequent administration of galactosylated clearing antibody might result the two systems acting synergistically to give greater enhancement of tumour:normal tissue ratios and hence improve the efficacy of the ADEPT system in antitumour trials. The experiments described here were performed in a syngeneic animal/tumour model and an obvious further step would be to test the same system in the LS174T colon cancer/nude mouse model.


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