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Ablation of Human Choriocarcinoma Xenografts in Nude Mice by Antibody-directed Enzyme Prodrug Therapy (ADEPT) with Three Novel Compounds

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Three novel prodrugs have been designed for use as anticancer agents. Each is a bifunctional alkylating agent which has been protected to form a relatively inactive prodrug. They are designed to be activated to their corresponding alkylating agents at a tumour site by prior administration of an antitumour antibody conjugated to the bacterial enzyme carboxypeptidase G2 (CPG2) in a two-phase system called antibody-directed enzyme prodrug therapy (ADEPT). The K_m and V_{max} values for three different antibody–CPG2 conjugates were determined in relation to each prodrug. The K_m values ranged from 4.5–12 μmol/l and the V_{max} from 0.5–1.6 μmol/U/min. Athymic *Nu/Nu* mice with palpable transplanted human choriocarcinoma xenografts, which are resistant to conventional chemotherapy, were treated with anti-human chorionic gonadotropin antibodies conjugated to CPG2. This was followed by each of the three novel prodrugs. Significant increase in survival was obtained in three of the regimens tested using only one course of treatment. This demonstrates the potential of a tumour-localised bacterial enzyme to activate protected alkylating agents in order to eradicate an established human xenograft.
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INTRODUCTION

CANCER CHEMOTHERAPY is hampered by the low therapeutic index of most anticancer drugs. Selective generation of a cytotoxic active drug from an inactive prodrug at the tumour site has become, therefore, an important goal. Ideally, the drug, once generated, should interact quickly at the site of its formation, for if it exhibits slow uptake at the tumour and/or has high diffusibility away from the tumour the advantage will be lost [1].

Antibody-directed enzyme prodrug therapy (ADEPT) [2, 3] separates the cytotoxic function from the targeting function in a two-phase system which has several benefits over a one-phase chemo- or radioimmunoconjugate. Radioisotopes linked to antibodies cause normal tissue cytotoxicity during their clearance phase and before their preferential retention in tumours can dominate the distribution pattern. With chemoimmunoconjugates only a limited number of cytotoxic molecules can be linked to an antibody molecule, internalisation of the drug–antibody conjugate may present problems and it is necessary to achieve targeting to most cells in the tumour mass. The amplification inherent in the enzyme component of an antibody–enzyme conjugate may compensate for the low proportion of administered antibody retained in tumours *in vivo*. Alkylating agents are good candidates for ADEPT in that their cytotoxicity is dose-related and they can be given repeatedly with less induced resistance than other classes of anticancer agents [4].

A series of three novel prodrugs 4-[*bis*(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid, 4-[2-chlorethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid and 4-[*bis*(2-chloroethyl)amino]benzoyl-L-glutamic acid has been synthesised [5] for use in ADEPT. Each drug is a bifunctional alkylating agent in which the activating effect of the ionised

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carboxyl function has been masked to form relatively inactive prodrugs.

Previous work has already established that anti-human chorionic gonadotropin antibodies conjugated to the enzyme CPG2 are preferentially retained in choriocarcinoma xenografts in nude mice [6]. CPG2 cleaves various folates and methotrexate to release glutamic acid [7, 8] and performs a similar hydrolysis of the amide bond in the prodrugs to form activated alkylating agents. The K_m and V_{max} values of the anti-human chorionic gonadotropin CPG2 (anti-hCG-CPG2) conjugates for each prodrug substrate have been determined and are of the same order as folic acid and methotrexate.

We present here a comparison of the growth delay of choriocarcinoma xenografts occasioned by treating the tumours with anti-hCG-CPG2 conjugates paired with each of the above prodrugs. The relationships between the efficacy of growth delay and the properties of each prodrug are discussed.

MATERIALS AND METHODS

Prodrugs

The three prodrugs, 4-[*bis*(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]-benzoyl-L-glutamic acid and 4-[*bis*(2-chloroethyl) amino]-benzoyl-L-glutamic acid were prepared as described previously [5]. The structures of the three prodrugs with their respective activated drugs are shown in Fig. 1.

Monoclonal antibodies

The W14A and SB10 antibodies are monoclonals raised against the β-subunit of human chorionic gonadotropin [9, 10]. Pepsin fragmentation and subsequent purification followed established procedures [11]. The antibody A5B7, an anti-human carcinoembryonic antigen mouse monoclonal [12], was fragmented in a similar manner and was used as a non-specific antibody with the choriocarcinoma xenograft.

Conjugation

Conjugation of the W14A $F(ab')_2$ fragment (W14) to CPG2 was achieved in a manner similar to that described for the intact antibody. This used N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP)-thiol-enriched antibody and 4-(P-maleimidophenyl)butyric acid N-hydroxysuccinimide ester (MBS) modified enzyme to give the conjugate designated W14 = CP [6, 8]. Conjugation of the SB10 $F(ab')_2$ fragment (S10) to give S10 = CP conjugate was achieved via a modification of this method in which S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was used for insertion of thiol residues. The A5B7 $F(ab')_2$ fragment (A5) was conjugated to CPG2 to give A5 = CP as described for S10 = CP. All preparations produced conjugates linked by a stable thio-ether bond. The W14 = CP and A5 = CP were predominantly 1:1 whereas the S10 = CP was predominantly 1:2 $F(ab')_2$-enzyme conjugates, as determined by their elution positions on gel filtration columns of Superose 6 in fast protein liquid chromatography (Pharmacia).

Michaelis–Menten kinetics

The K_m and V_{max}, calculated using each of the prodrugs with the W14 = CP, S10 = CP or the A5 = CP conjugate, was determined by a modification of the CPG2 assay method for methotrexate [14]. Each conjugate was first assayed in a reaction mixture (1 ml) which contained Tris-HCl (pH 7.3, 100 μmol), $ZnCl_2$ (260 nmol) and methotrexate (60 nmol) at 37°C. The reaction was initiated by the addition of enzyme conjugate (1–10 U) and the change in absorbance at 320 nm recorded on a double beam spectrophotometer (SP8-150 Pye Unicam, Cambridge, UK) fitted with a cell temperature controller. A value of 8300 litre/mol/cm was used for the molar extinction coefficient change of methotrexate at 320 nm. One unit (U) of enzyme activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μmol of methotrexate/min/ml under the above reaction conditions.

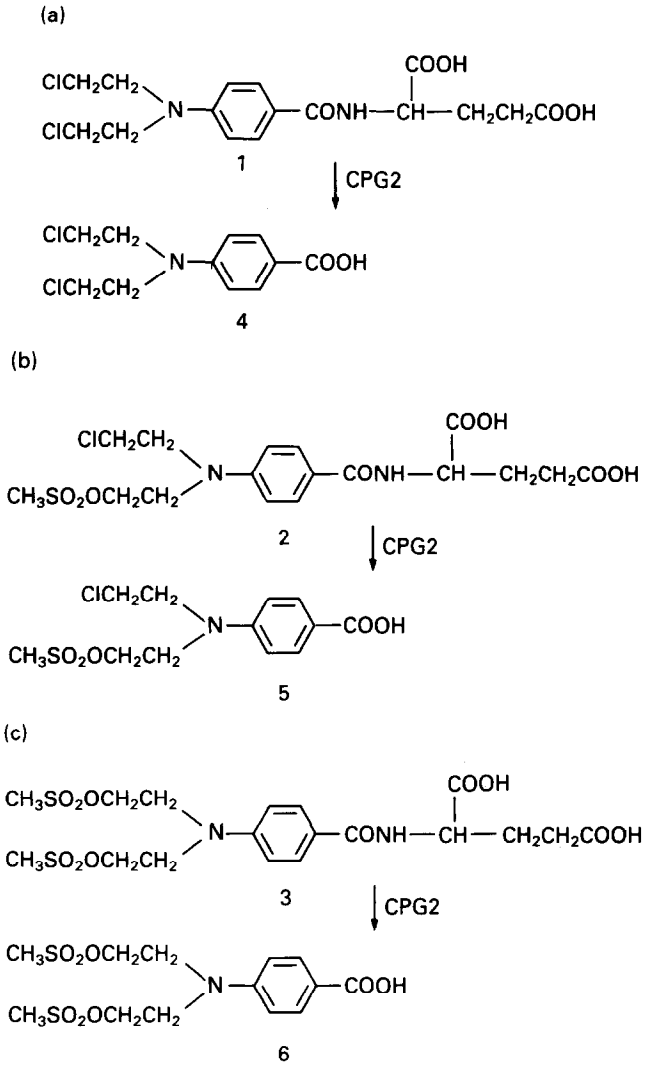

Fig. 1. (a) The structure of 4-[*bis*(2-chloroethyl)amino]benzoyl-L-glutamic acid [1] and its activated drug [4]; (b) the structure of 4-[2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid [2] and its activated drug [5]; (c) the structure of 4-[*bis*(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid [3] and its activated drug [6].

On addition of CPG2 to each prodrug there is a decrease in the absorption spectrum, as a result of the hydrolysis of the glutamic acid moiety, which leads to the formation of the active parent drug. The molar extinction change at 320 nm was measured by determining the absorption of a known concentration of each prodrug and then of the corresponding active drug by addition of CPG2. The K_m and V_{max} were obtained as for the CPG2 assay of methotrexate above except that prodrug, at nine different dilutions in the concentration range 0.71–14.60 μmol/l, was substituted for methotrexate and W14 = CP, S10 = CP or A5 = CP (0.003–0.016 U) were used in place of CPG2. Plots of initial reaction velocity versus each prodrug substrate concentration followed Michaelis–Menten kinetics.

Table 1. Kinetic properties of the prodrugs

Prodrug	W14 = CP		S10 = CP		A5 = CP	
	K_m*	V_{max}†	K_m*	V_{max}†	K_m*	V_{max}†
4-[*bis*(2-mesyloxyethyl)amino] benzoyl-L-glutamic acid	9.0 (1.4)	0.97 (0.10)	10 (1.6)	0.58 (0.07)	10 (2.9)	1.42 (0.29)
4-[(2-chloroethyl)(2-mesyloxyethyl) amino]benzoyl-L-glutamic acid	5.1 (1.5)	0.51 (0.09)	12 (2.5)	0.68 (0.10)	7.7 (1.6)	0.75 (0.09)
4-[*bis*(2-chloroethyl)amino] benzoyl-L-glutamic acid	4.5 (0.7)	0.62 (0.04)	12 (1.9)	1.55 (0.19)	5.4 (0.8)	1.14 (0.11)

Mean (S.E.).
* μmol/l, † μmol/U/min.
K_m = the Michaelis constants for each prodrug with each conjugate, determined spectrophotometrically by a plot of the change in initial reaction velocity for 9 different dilutions of prodrug versus the prodrug substrate concentration.

Therapy schedules

The CC3 human choriocarcinoma xenograft was passaged in male nude mice as described previously [8]. Animals were included in the experiment when the tumours were just palpable, i.e. when volumes = $0.1 < (dl \times d2 \times d3 \div 2) < 0.35$ cm^3 [15] and the serum human chorionic gonadotropin (hCG) measurements were between 100 and 1000 IU/litre. hCG levels were determined from diluted mouse serum (1/20) on the Kemtek automated radioimmunoassay system with a sensitivity limit of detection of 2 IU/litre [16]. Conjugates were then administered to the xenograft-bearing nude mice and serum CPG2 levels were measured by the standard spectrophotometric assay for methotrexate cleavage (as detailed above). Prodrug was administered only when the serum CPG2 level was < 0.40 U/ml, according to the following schedules.

4-[bis(2-chloroethyl)amino]benzoyl-L-glutamic acid. Groups of four animals received either 10, 25 or 50 U of W14 = CP as a single intravenous injection. The prodrug was administered as 3 × 5 mg or 3 × 10 mg intravenous injections at time 0, 22 and 27 h. The time of the first prodrug injection was adjusted such that it was delivered 24, 48, 56 or 72 h after the conjugate injection. The prodrug was made up in dimethyl sulphoxide/phosphate-buffered saline (1:16) immediately prior to injection. Control animals received equal volumes of saline to test animals.

4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid. Groups of six animals received either 50 U of W14 = CP or S10 = CP followed 72 h later by prodrug (3 × 10 mg) made up and administered as above. Groups of control animals received either 50 U of A5 = CP followed 72 h later by prodrug (3 × 10 mg) or prodrug alone (3 × 10 mg) or equal volumes of saline to test animals.

4-[bis(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid compared to 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]-benzoyl-L-glutamic acid. Two groups of six animals each received 10 U of S10 = CP followed 24 h later by equimolar concentrations of each prodrug—either the chloroethylmesyloxyethyl (3 × 5 mg) or the *bis*-mesyloxyethyl prodrug (at an equimolar concentration of 3 × 5.6 mg) as above. Control animals received equal volumes of saline to test animals.

In all experiments, tumour volumes were measured and animals culled when the tumour had reached a volume $(dl \times d2 \times d3 \div 2) = 2$ cm^3 [15]. At the end of the experiment in animals where ADEPT treatment had led to successful xenograft remissions, hCG levels in blood were measured.

Data analysis. A two-sided logrank test [17] was performed to assess whether the survival times for each treatment group were similar to the survival times for the associated control group. A low P value indicates a difference between the treatment group and the control group.

RESULTS

CPG2 conjugated to the F(ab')$_2$ fragments of W14A, SB10 or A5B7 readily cleaves the glutamic acid moiety from each prodrug, as shown in Table 1. All three prodrugs were found to be good substrates for all conjugates, although there were minor differences in substrate specificity for each conjugate. The K_m values using all three prodrugs with the S10 = CP were in close range (10–12 μmol/l). However, there was a two-fold difference in the range of K_m values with W14 = CP, from 4.5 μmol/l for the *bis*chloroethyl prodrug, 5.1 μmol/l for the chloroethylmesyloxyethyl prodrug and 9.0 μmol/l for the *bis*mesyloxyethyl prodrug. There was also a difference of two-fold in the range of K_m values with the A5 = CP from 5.4 μmol/l for the *bis*chloroethyl prodrug to 10 μmol/l for the *bis*mesyloxyethyl prodrug. The chloroethylmesyloxyethyl prodrug had an intermediate K_m of

Table 2. Extension of survival of CC3-bearing nude mice in response to conjugated enzyme plus the bischloroethyl prodrug

Group	Enzyme conjugate (U)	Administration of prodrug—interval (h) postconjugate	Prodrug dose (mg) × 3 per animal	P*
1	10	24	5	0.101
2	10	56	5	0.254
3	25	48	5	0.298
4	25	56	5	0.150
5	25	72	5	0.088
6	50	56	10	0.101
7	50	72	10	0.027†

Groups 1–7 (4 animals/group) received W14 = CP intravenously followed by prodrug intravenously at differing time intervals after the conjugate injections.
* vs. control group (logrank test). † Significant difference.

Table 3. Extension of survival in response to conjugated enzyme plus the chloroethylmesyloxyethyl prodrug

Group	Enzyme conjugate type and dose (U)	Administration of prodrug—interval (h) postconjugate	Prodrug dose (mg) × 3 per animal	*P*
8	S10 = CP 50	72	10	*0.0009
9	W14 = CP 50	72	10	*0.00006

Groups 8–9 (6 animals/group) received either S10 = CP or W14 = CP intravenously followed by prodrug intravenously.
* Significant difference.

Table 4. Extension of survival in response to irrelevant conjugated antibody, A5 = CP plus the chloroethylmesyloxyethyl prodrug, or prodrug alone

Group	Enzyme conjugate (U)	Administration of prodrug—interval (h) postconjugate	Prodrug dose (mg) × 3 per animal	*P*
10	0	—	10	0.390
11	50	72	10	0.186

Control group 10 (4 animals/group) received prodrug intravenously without prior conjugate. Control group 11 (4 animals/group) received the irrelevant antibody conjugate A5 = CP followed by prodrug intravenously.

7.7 μmol/l. The V_{max} figures exhibit a range of values from 0.51–1.55 μmol/U/min.

Evidence of the antitumour activities of the prodrugs *in vivo* in ADEPT is shown in Tables 2–5 and in Figs 2–5. The *bis*chloroethyl prodrug was the most extensively tested. Three different doses of conjugate were used in combination with two dose schedules of prodrug with four different time intervals. Extension of survival beyond control animals was significant in one group of animals that received 50 U of conjugate with an interval of 72 h prior to 3 × 10 mg prodrug (group 7, Table 2; Fig. 2h).

There was significantly greater survival over controls with the chloroethylmesyloxyethyl prodrug under the same conditions with both relevant conjugates (groups 8 and 9, Table 3; Fig. 3b, c) using 50 U conjugate with 3 × 10 mg prodrug starting 72 h postconjugate. With W14 = CP, five out of six animals were long-term survivors (group 9, Table 3; Fig. 3c), whilst with S10 = CP (group 8, Table 3; Fig. 3b) four out of six survived, until the experiment was terminated (the first animal in this group died of an infection which was independent of the tumour xenograft). The statistics showed significance in both groups, with values of $P < 0.0001$ and $P < 0.001$ for the W14 = CP and the S10 = CP, respectively. Control animals given the chloroethylmesyloxyethyl prodrug without prior conjugate showed no significant difference from controls (group 10, Table 4; Fig. 4b).

Table 5. Comparison of survival in response to conjugated enzyme plus the chloroethylmesyloxyethyl prodrug or the bismesyloxyethyl prodrug, at low levels of enzyme

Group	Enzyme conjugate (U)	Administration of prodrug—interval (h) postconjugate	Prodrug type and dose (mg) × 3 per animal	*P*
12	10	24	chloroethyl mesyloxyethyl 5	0.105
13	10	24	*bis*mesyloxyethyl 5.6	0.636

Groups 12 and 13 (6 animals/group) received S10 = CP intravenously followed by equimolar amounts of two types of prodrug intravenously at suboptimal doses.

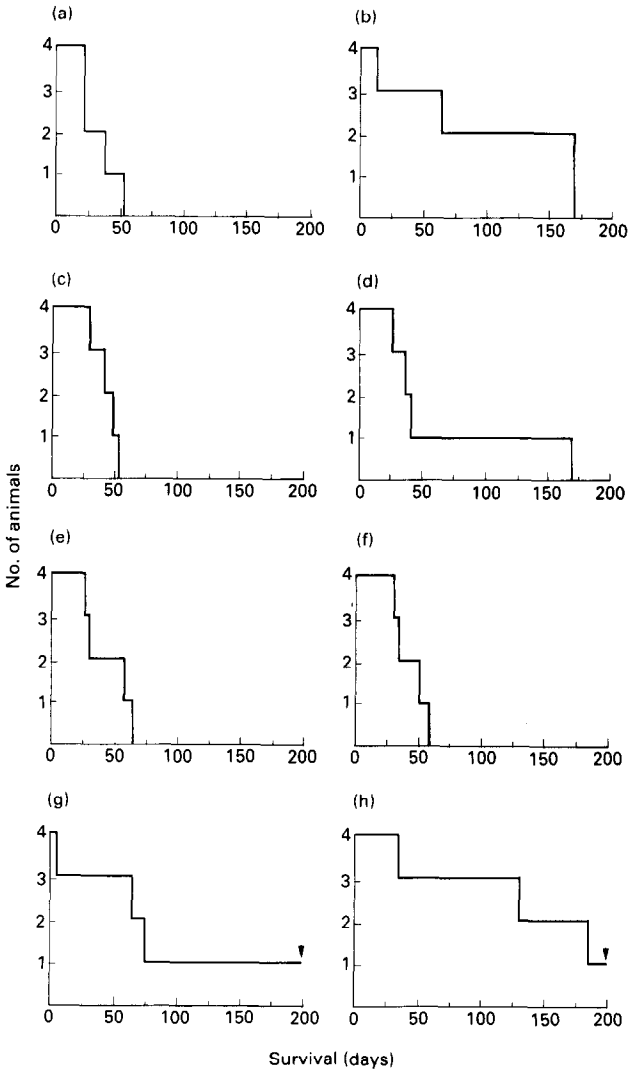

Fig. 2. Extension of survival of CC3-bearing nude mice in response to conjugated enzyme plus the bischloroethyl prodrug. (a) Control group which received saline; (b) test group 1, which received W14 = CP (10 U) followed 24 h later by the prodrug (3 × 5 mg); (c) test group 2, which received W14 = CP (10 U) followed 56 h later by the prodrug (3 × 5 mg); (d) test group 3, which received W14 = CP (25 U) followed 48 h later by the prodrug (3 × 5 mg); (e) test group 4, which received W14 = CP (25 U) followed 56 h later by the prodrug (3 × 5 mg); (f) test group 5, which received W14 = CP (25 U) followed 72 h later by the prodrug (3 × 5 mg); (g) test group 6, which received W14 = CP (50 U) followed 56 h later with the prodrug (3 × 10 mg); and (h) test group 7, which received W14 = CP (50 U) followed 72 h later with the prodrug (3 × 10 mg). All injections administered intravenously. ▼ = Experiment terminated.

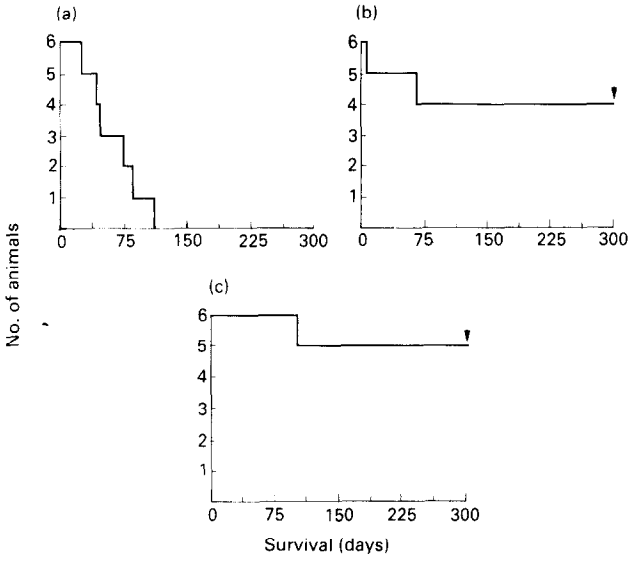

Fig. 3. Extension of survival of CC3-bearing nude mice in response to conjugated enzyme plus the chloroethylmesyloxyethyl prodrug. (a) Control group which received saline; (b) test group 8, which received S10 = CP (50 U) followed 72 h later with the prodrug (3 × 10 mg); (c) test group 9, which received W14 = CP (50 U) followed 72 h later with the prodrug (3 × 10 mg). ▼ = Experiment terminated.

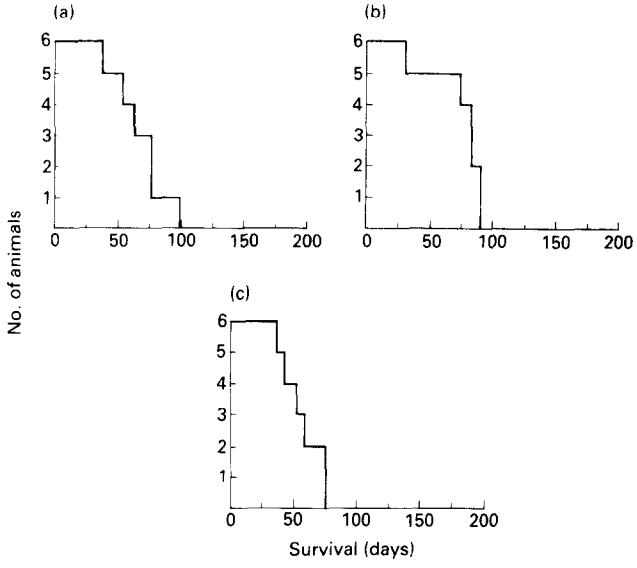

Fig. 5. Comparison of extension of survival of CC3-bearing nude mice in response to conjugated enzyme plus the chloroethylmesyloxyethyl prodrug or the bismesyloxyethyl prodrug at equimolar concentrations and at low levels of enzyme. (a) Control group which received saline; (b) test group 12, which received S10 = CP (10 U) followed 24 h later with the chloroethylmesyloxyethyl prodrug (3 × 5 mg); (c) test group 13, which received S10 = CP (10 U) followed 24 h later with the *bis*mesyloxyethyl prodrug (3 × 5.6 mg).

Animals that received 50 U of the irrelevant antibody conjugate, A5 = CP, followed by 3 × 10 mg prodrug starting 72 h postconjugate did not have a significant difference compared to controls (group 11, Table 4; Fig. 4c). In animals that showed complete ablation of tumour xenografts with the ADEPT therapy, hCG could not be detected in the blood at the termination of the experiments.

The *bis*mesyloxyethyl prodrug was tested only under limited conditions at low levels of enzyme-conjugate to determine whether it would perform better than the other two prodrugs (group 13, Table 5; Fig. 5c). Since there was no improvement when compared to the chloroethylmesyloxyethyl prodrug (group 12, Table 5; Fig. 5b), no further *in vivo* therapies were performed on this prodrug.

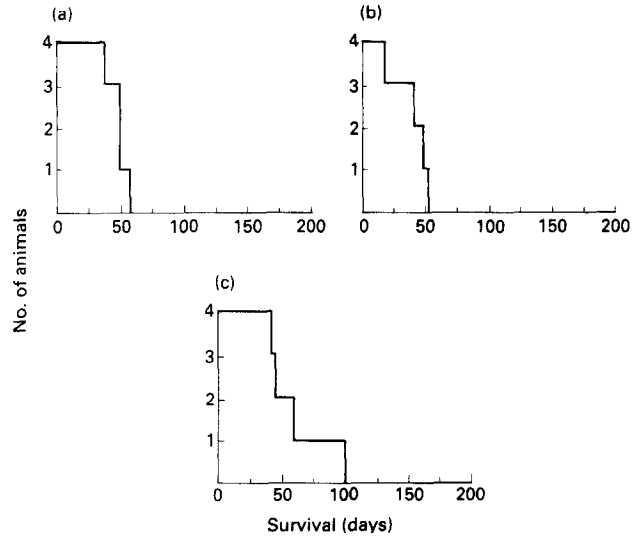

Fig. 4. Extensions of survival of CC3-bearing nude mice in response to irrelevant conjugated antibody, A5 = CP plus the chloroethylmesyloxyethyl prodrug, or prodrug alone. (a) Control group which received saline; (b) test group 10, which received prodrug alone (3 × 10 mg); (c) test group 11, which received A5 = CP (50 U) followed 72 h later with the prodrug (3 × 10 mg).

DISCUSSION

We have previously shown that when a monoclonal antibody-enzyme immunoconjugate is administered prior to the *bis*chloroethyl prodrug in ADEPT, transplanted resistant choriocarcinoma tumours are eradicated in nude mice *in vivo* [2, 18, 19]. The studies presented here indicate that under optimised conditions the chloroethylmesyloxyethyl prodrug gives enhanced survival of nude mice with transplanted choriocarcinoma xenografts. Another group has also used an ADEPT approach to obtain antitumour responses, but with etoposide and mitomycin prodrugs [20, 21].

A comparison of the kinetics of the prodrugs with the three conjugates shows two different trends. In the cases of the W14 = CP and A5 = CP conjugates, there is a decrease in K_m with decreasing size, from the largest prodrug, the *bis*mesyloxyethyl, to the smallest, the *bis*chloroethyl compound. The W14 = CP and A5 = CP conjugates both consisted of one enzyme molecule per $F(ab')_2$ fragment in contrast to the S10 = CP which consisted predominantly of two enzyme molecules per $F(ab')_2$ fragment. The K_m values with the latter conjugate gave a similar result for each prodrug irrespective of the size of the alkylating moiety of the prodrugs. The differences in K_m values may be due to an increase in steric hindrance where there is a 1:2 ratio of $F(ab')_2$ to enzyme as in S10 = CP. It should be noted that all the K_m values obtained for the three prodrugs compare favourably with the reported values of 8.0 μmol/l for methotrexate with CPG2 [14]. These kinetic studies demonstrate that all three prodrugs are suitable substrates for use in ADEPT with the CPG2 enzyme.

The *bis*chloroethyl prodrug was tested under various different regimens in an attempt to optimise the conditions for ADEPT. Previous work in the same xenograft model had demonstrated that the tumour growth was not significantly delayed in the

presence of conjugate alone or of the maximum tolerated doses of the *bis*chloroethyl prodrug or its activated drug [2]. Nor did it respond to courses of the conventional chemotherapeutic agents methotrexate, hydroxyurea, actinomycin D, cyclophosphamide or cytarabine [2]. When the chloroethylmesyloxyethyl prodrug was tested under the conditions which were found to be optimal for the *bis*chloroethyl prodrug, the preliminary growth delay seen previously with the latter compound was confirmed. However, the long-term results were more consistent with the chloroethylmesyloxyethyl prodrug. Growth of CC3 xenografts in *Nu/Nu* mice was substantially delayed by administration of 50 U of the enzyme CPG2 conjugated to anti-hCG $F(ab')_2$ antibody fragments, followed 72 h later by 3×10 mg doses of the chloroethylmesyloxyethyl prodrug over 27 h. Nine out of 12 animals were long-term survivors, whereas all control animals were dead by day 111.

In order to establish the potency of the chloroethylmesyloxyethyl prodrug, a third compound, the *bis*mesyloxyethyl prodrug, was compared under a regimen using suboptimal doses of enzyme conjugate and prodrug. However, despite the similar kinetics of the *bis*mesyloxyethyl prodrug for the enzyme conjugates it did not prove to be as effective at enhancing suvival of the test animals.

Sustained levels of the activated drug of the *bis*chloroethyl prodrug at the tumour site indicate that the active drug released at the tumour site is generated by localised enzyme [22]. In the therapy regimens adopted here, the residual plasma enzyme levels and the time interval between conjugate and prodrug administrations were critical factors for effective therapy. Animals given 10 mg $\times$ 3 doses of the *bis*chloroethyl prodrug where plasma enzyme levels were > 0.4 U/ml died soon after administration due to non-localised release of activated drug. When 10 U of antibody–enzyme conjugate was administered, *bis*chloroethyl prodrug treatment could begin 24 h later. However, when 25 U of enzyme conjugate was injected, *bis*chloroethyl prodrug treatment could not start until 48 h later in order to avoid toxicity due to activated drug from residual plasma enzyme conjugate. Administration of 50 U of enzyme conjugate needed the greater time interval of 56 h, but a greater amount of prodrug could then be administered, leading to significant difference in survival over controls.

The balance between enzyme level at the tumour and in plasma is critical, leading in turn to the differential release of the activated drug. It has been shown that a considerably higher proportion of prodrug is cleared through plasma and liver than will arrive at the tumour [22], and therefore a low level of enzyme in the plasma may effect as much turnover of prodrug as a high concentration of enzyme in the more limited volume of interstitial fluid at the tumour site. But when time is allowed for non-tumour localised conjugate to clear before administration of a prodrug substrate with a high affinity for the enzyme, a substantial level of drug can be activated at the tumour site.

Studies are in progress to optimise further the antibody-directed enzyme prodrug therapy described here. It is very encouraging to have observed substantially enhanced survival in mice with choriocarcinoma xenografts which are resistant to all conventional chemotherapeutic agents tested, in response to only one course of treatment with the chloroethylmesyloxyethyl prodrug in ADEPT.

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