

Efficient Clearance of Poly(ethylene glycol)-Modified Immunoenzyme with Anti-PEG Monoclonal Antibody for Prodrug Cancer Therapy

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The F(ab')₂ fragment of the anti-TAG-72 antibody, B72.3, was covalently linked to *Escherichia coli*-derived β -glucuronidase that was modified with methoxypoly(ethylene glycol). The conjugate (B72.3- β G-PEG) localized to a peak concentration in LS174T xenografts within 48 h after injection, but enzyme activity persisted in plasma such that prodrug administration had to be delayed for at least 4 days to avoid systemic prodrug activation and associated toxicity. Conjugate levels in tumors decreased to 36% of peak levels at this time. Intravenous administration of AGP3, an IgM mAb against methoxypoly(ethylene glycol), accelerated clearance of conjugate from serum and increased the tumor/blood ratio of B72.3- β G-PEG from 3.9 to 29.6 without significantly decreasing the accumulation of conjugate in tumors. Treatment of nude mice bearing established human colon adenocarcinoma xenografts with B72.3- β G-PEG followed 48 h later with AGP3 and a glucuronide prodrug of *p*-hydroxyaniline mustard significantly ($p \leq 0.0005$) delayed tumor growth with minimal toxicity compared to therapy with a control conjugate or conventional chemotherapy.

INTRODUCTION

A major goal of antitumor drug development is to increase the therapeutic index of chemotherapy, thereby improving treatment efficacy. One approach to increase the therapeutic index of chemotherapy is to preferentially activate antineoplastic prodrugs at cancer cells but not normal tissues. Tumor selectivity may be achieved by enzymatically converting prodrugs possessing low toxicity to highly toxic anti-neoplastic agents by previously administered antibody-enzyme conjugates (immunoenzymes) that have been allowed to accumulate at tumor cells (Bagshawe et al., 1988; Senter et al., 1988). Even though maximum accumulation of immunoenzymes in tumors occurs around 24 h after administration (Bosslet et al., 1994; Wallace et al., 1994), prodrugs are generally administered from 3 to 7 days (Bosslet et al., 1994; Svensson et al., 1998) to up to 2 weeks (Eccles et al., 1994) later to allow adequate time for conjugate to clear from the blood, thereby minimizing systemic prodrug activation and associated toxicity to normal tissues. Although exceptions have been reported for rapidly clearing immunoenzymes (Siemers et al., 1997), the requisite of low circulating levels of immunoenzyme often precludes prodrug administration when maximum localization has been achieved.

Prodrugs can be administered during the period of maximum tumor accumulation of immunoenzymes if circulating conjugates are removed or deactivated. Several methods have been devised to accelerate the clearance of radioimmunoconjugates and immunoenzymes

from the circulation including the administration of polyclonal (Stewart et al., 1990) and anti-idiotypic antibodies (Ullen et al., 1995) against the antibody portion of the immunoconjugate, injection of avidin to clear biotinylated antibodies (Paganelli et al., 1991), use of monoclonal antibodies against enzymes to clear (Kerr et al., 1993; Haisma et al., 1995) or deactivate (Sharma et al., 1990) immunoenzymes, and extracorporeal immunoadsorption (Tennvall et al., 1997) to remove immunoconjugates from plasma.

We have recently developed a mAb¹ (AGP3) that binds to poly(ethylene glycol) (PEG) and accelerates the clearance of immunoconjugates that have been modified with PEG (Cheng et al., 1999b). PEG-modified proteins often exhibit extended serum half-lives, reduced immunogenicity, and decreased susceptibility to proteolytic degradation (Delgado et al., 1992). Antibody fragments and immunoconjugates that have been modified with PEG also display reduced normal tissue uptake and enhanced tumor accumulation (Pedley et al., 1994; Delgado et al., 1996; Cheng et al., 1997). Clearance of PEG-modified immunoconjugates with AGP3 may, therefore, be generally useful. In the present study, we examined whether AGP3 could improve tumor-blood ratios and allow earlier administration of prodrug in a preclinical model of human colorectal carcinoma. PEG-modified β G was covalently linked to the F(ab')₂ fragment of mAb B72.3, an

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¹ Abbreviations: β G, β -glucuronidase derived from *E. coli*; β G-PEG, β -glucuronidase modified with methoxypoly(ethylene)glycol; pHAM, *p*-hydroxyaniline mustard; BHAMG, tetra *n*-butylammonium salt of the glucuronide of *p*-hydroxyaniline mustard; mAb, monoclonal antibody; B72.3- β G-PEG, conjugate of the F(ab')₂ fragment of mAb B72.3 with β G-PEG; H25- β G-PEG, conjugate of the F(ab')₂ fragment of mAb H25B10 with β G-PEG; TAG-72, tumor-associated glycoprotein; PNPG, *p*-nitrophenol β -D-glucuronide; IC₅₀, drug concentration causing 50% inhibition of cellular protein synthesis.

IgG₁ antibody that binds to TAG-72 antigen expressed on the majority of colon adenocarcinomas, invasive ductal carcinomas of the breast, nonsmall cell lung carcinomas, common epithelial ovarian carcinomas, and gastric, pancreatic, and esophageal cancers with limited reactivity to normal adult tissues (Thor et al., 1986). mAb B72.3 is approved for clinical use as an imaging agent (Oncosint CR/OV) for colorectal and ovarian carcinoma (Divgi, 1996). We show that clearance of a PEG-modified B72.3 immunoconjugate with AGP3 produced higher tumor-blood ratios without sacrificing tumor accumulation, allowing earlier prodrug administration with minimal toxicity.

MATERIALS AND METHODS

Reagents. The syntheses of pHAM and BHAMG have been described (Roffler et al., 1991). Bolton–Hunter reagent (¹²⁵I) was purchased from Amersham (Buckinghamshire, England). Succinimidyl succinate poly(ethylene glycol), MW = 5000, *p*-nitrophenyl- β -D-glucuronide, and Sepharose CL-4B protein A were purchased from Sigma Chemical Company, St. Louis, MO. Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate (SMCC) was from Pierce Chemical Company, Rockford, IL. Sephadex G-25 and Sephacryl S-300 HR gels were purchased from Pharmacia Biotech Far East Ltd., Taipei, Taiwan. Recombinant β G was produced as described (Cheng et al., 1997).

Cells. LS174T colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated bovine serum, 100 units/mL penicillin and 100 μ g/mL streptomycin. Cells were routinely tested for mycoplasma with the Rapid Detection Kit according to the manufacturer's instructions (Gene Probe).

Animals. BALB/c mice were obtained from the animal room of the Institute of Biomedical Sciences, Academia Sinica. BALB/c *nu/nu* mice were from the Cancer Research Laboratory, Tri-Service General Hospital, Taipei. Animal experiments were performed in accordance with institute guidelines.

Antibodies. Hybridomas secreting mAb B72.3, an IgG₁ mAb specific for TAG-72 antigen (Johnson et al., 1986), and H25B10, a control IgG₁ mAb against the surface antigen of hepatitis B virus, were obtained from the American Type Culture Collection. mAbs were purified from ascites by affinity chromatography on Sepharose CL-4B protein A in high-salt buffer (Ey et al., 1978). F(ab')₂ fragments were generated by proteolytic digestion of whole antibodies (2–5 mg/mL) with 2% bromelain in 50 mM Tris-HCl, pH 7.0, containing 2 mM EDTA and 0.1 mM cysteine for 4 h at 37 °C (Milenic et al., 1989). After stopping the reaction by addition of *N*-ethylmaleimide to 10 mM, the mixture was passed in series through Sephadex G-50 and DE 52 columns equilibrated with 5 mM Tris-HCl, pH 7.5, to remove IgG and Fc fragments. The flow through fraction was concentrated to 5 mg/mL and purified by gel filtration on Sephacryl S-200HR (100 \times 2.5 cm) equilibrated with PBS. AGP3, an IgM mAb that binds to PEG (Cheng et al., 1999b), was purified from ascites by gel filtration on Sephacryl S-400HR (2.5 \times 100 cm) equilibrated with PBS.

Antibody- β G-PEG Conjugates. F(ab')₂ fragments of mAb B72.3 and H25B10 were passed through a 2.6 \times 30 cm Sephadex G-25 column equilibrated with coupling buffer (deoxygenated PBS containing 1 mM EDTA, pH 8.0) and concentrated by ultrafiltration to 3.0 mg/mL. A

3-fold molar excess of SMCC (1 mg/mL in dioxane) was slowly added to the antibodies and allowed to react at room temperature for 50 min. Unreacted SMCC was removed by gel filtration on a 2.6 \times 30 cm Sephadex G-25 column equilibrated with coupling buffer. The average number of maleidido groups introduced into antibodies (0.8–1.4) was assessed as described (Ishikawa et al., 1987). Succinimidyl succinate poly(ethylene glycol) was added to recombinant β G derived from *Escherichia coli* (2 mg/mL) at a weight ratio of 3.3:1 in coupling buffer at room temperature for 2 h. One-tenth volume of a saturated solution of glycine in coupling buffer was added to stop the reaction. β G-PEG was concentrated by ultrafiltration to 1.5 mg/mL and partially reduced by adding dithiothreitol to a final concentration of 20 mM for 30 min at room temperature. β G-PEG was desalted on a 2.6 \times 30 cm Sephadex G-25 column equilibrated with coupling buffer and concentrated to 1.5 mg/mL. Freshly derivatized antibody and β G-PEG were immediately mixed at equal molar ratios, concentrated to 1.5 mg/mL by ultrafiltration, and incubated at room temperature for 2 h. Cysteine was added to a final concentration of 2 mM to stop the reaction, and the mixture was concentrated by ultrafiltration to 5–8 mg/mL. Conjugates (B72.3- β G-PEG and H25- β G-PEG) were purified by gel filtration on a 2.6 \times 100 cm Sephacryl S-300 HR column equilibrated with PBS at a flow rate of 15 mL/h. Fractions containing conjugate monomers (antibody/enzyme = 1:1) were pooled and concentrated by ultrafiltration to 2.0 mg/mL for storage at –80 °C. The yield of conjugates averaged around 31% based on the weight of purified conjugate divided by the total starting weights of antibody and enzyme. The antigen-binding activity of conjugates was measured by ELISA in 96-well microtiter plates coated with bovine submaxillary gland mucin (King et al., 1994). β G activity was measured as described (Chen et al., 1997).

Drug Sensitivity. LS174T cells were plated overnight in 96 well microtiter plates at 40 000 cells/well. Serial dilutions of pHAM or BHAMG in medium containing 10% fetal calf serum were added to cells in triplicate for 24 h at 37 °C. Cells were subsequently washed once with sterile PBS, incubated until hour 48 in fresh medium, and then pulsed for 12 h with [³H]leucine (1 μ Ci/well) in fresh leucine-free medium. Cells were harvested with a Filter-mate apparatus (Packard) and incorporated radioactivity was determined on a Top-Count scintillation counter (Packard). Results are expressed as percentage inhibition of [³H]leucine incorporation compared with untreated cells by the following formula:

$$\% \text{ inhibition} = 100 \times \frac{\text{cpm sample} - \text{cpm background}}{\text{cpm control} - \text{cpm background}}$$

Radiolabeling of Conjugates. B72.3- β G-PEG and H25- β G-PEG were labeled with [¹²⁵I]Bolton–Hunter reagent to specific activities of 0.3–0.7 μ Ci/ μ g according to the manufacturer's instructions. Conjugates retained antigen-binding activity and specificity as determined by radioimmunoassay against bovine submaxillary gland mucin in 96-well microtiter plates. β G activity was unaffected and conjugates were not degraded as determined by autoradiography of gels after sodium dodecyl sulfate–polyacrylamide electrophoresis.

Tumor Localization of B72.3- β G-PEG. A total of 5 \times 10⁶ LS174T cells were injected s.c. in 6–8 week old BALB/c *nu/nu* mice. After tumors reached 100–200 mm³, 200 μ g (60 μ Ci) of [¹²⁵I]B72.3- β G-PEG or [¹²⁵I]H25-

β G-PEG were i.v. injected into the lateral tail vein of mice. Groups of 3 mice were sacrificed after 24, 48, 72, and 96 h. Tumors, blood, and organs were weighed on an analytical balance and assayed for radioactivity in a multichannel γ -counter. Results are expressed as uptake of conjugate in tumor or tissues (% injected dose/gram).

In Vivo Clearance of Conjugates. Groups of two to three BALB/c *nu/nu* mice were i.v. injected with 250 μ g of B72.3- β G-PEG or H25- β G-PEG at time zero. Blood samples were periodically removed before two sequential i.v. injection of 300 and 200 μ g of AGP3 or PBS at 48 and 50 h. Additional blood samples were taken at subsequent times and the β G activity in duplicate samples was measured using *p*-nitrophenol β -D-glucuronide as substrate (Wang et al., 1992). Sample concentrations were calculated by comparison of absorbance values with a standard curve constructed from known concentrations of B72.3- β G-PEG or H25- β G-PEG.

Tumor Localization of B72.3- β G-PEG after Clearance. Groups of six to seven BALB/c *nu/nu* mice bearing 100–200 mm³ LS174T tumor xenografts were i.v. injected at time 0 with 200 μ g (140 μ Ci) [¹²⁵I]B72.3- β G-PEG or [¹²⁵I]-H25- β G-PEG. One-half of the mice was i.v. injected 48 and 50 h later with two sequential doses of 300 and 200 μ g of AGP3. Mice were sacrificed after 6 h, and tumor, blood, and organs were weighed on an analytical balance and assayed for radioactivity in a multichannel γ -counter. Urine sample were electrophoresed on a 3–12.5% SDS-PAGE under reducing conditions. Dried gels were exposed to X-ray film to visualize radioactive proteins.

Therapy of LS174T Tumor Xenografts. Groups of nine BALB/c *nu/nu* mice were s.c. injected on the right flank with 5×10^6 LS174T cells on day 1. On day 10, the mice were i.v. injected via the lateral tail vein with PBS, 250 μ g of B72.3- β G-PEG or H25- β G-PEG. Forty-eight and 50 h later, mice were i.v. injected with two sequential doses of 300 and 200 μ g of AGP3. Mice were i.v. injected 6 h later with three fractionated doses of 7.5 mg/kg BHAMG at 1 h intervals. Control groups of tumor-bearing mice were treated with BHAMG (7.5 mg/kg \times 3), pHAM (2 mg/kg \times 3) or PBS alone. Therapy was repeated starting on days 16, 26, and 41. All mice received two to four rounds of therapy. Tumor volumes (length \times width \times height \times 0.5) were estimated twice a week. Mean weight loss was normalized to the weight of control mice by weight loss % = $100 - [(T_i/T_0)(C_0/C_i)] \times 100$ where T_i and C_i are the mean weights of treated and control groups at time i , respectively, and T_0 and C_0 are the mean weights of treated and control groups before initiation of treatment, respectively. Mice were killed when they displayed signs of morbidity or when the tumor size exceeded 2.5 cm³. Groups of 8 BALB/c *nu/nu* mice bearing larger 200–250 mm³ tumors were also treated as above with two rounds of therapy starting on days 11 and 23.

Statistical Analysis. Statistical significance of differences between mean values was estimated with the shareware program Schoolstat (White Ant Occasional Publishing, West Melbourne, Australia) using the independent *t*-test for unequal variances.

RESULTS

Characterization of the LS174T Tumor Model. LS174T colon adenocarcinoma cells were used as a human tumor model to examine the effect of clearance on the localization and therapeutic efficacy of glucuronide prodrug activation by B72.3- β G-PEG, a conjugate formed by covalently linking F(ab')₂ fragments of mAb B72.3 to PEG-modified β G. Antigen-binding activity of B72.3- β G-

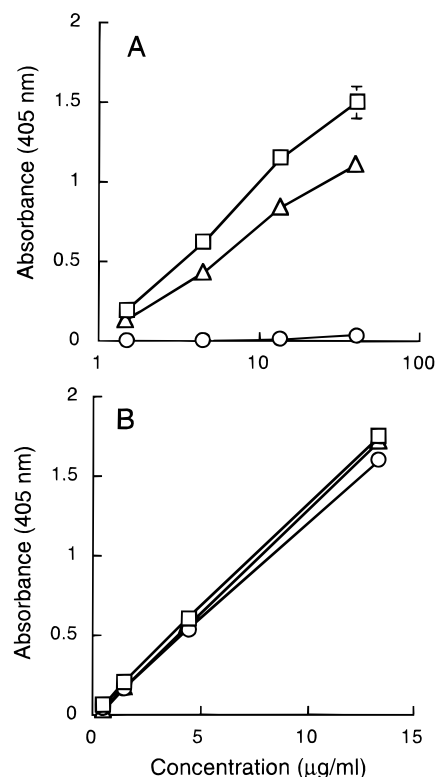


Figure 1. Immunoenzyme activity. (A) mAb B72.3 (□), B72.3- β G-PEG (Δ), and H25- β G-PEG (○) were assayed by ELISA for binding to bovine submaxillary gland mucin. Absorbance (405 nm) of wells was measured 30 min after addition of ABTS substrate. (B) The absorbance (405 nm) of wells containing the indicated concentrations of β G (□), B72.3- β G-PEG (Δ), and H25- β G-PEG (○) was measured 15 min after addition of PNPG substrate. Mean values of duplicate determinations are shown. Bars, SE.

PEG was assessed against bovine submaxillary gland mucin due to the low expression of TAG-72 antigen on cultured tumor cells (Horan Hand et al., 1985). B72.3- β G-PEG retained 75% of the mucin binding activity of mAb B72.3 (Figure 1A) and 100% of the enzyme activity of unmodified β G (Figure 1B). Control conjugate H25- β G-PEG did not bind mucin (Figure 1A) but retained 92.4% of native β G activity (Figure 1B). Immunohistochemical analysis of antibody binding confirmed that mAb B72.3 (Figure 2A) but not mAb H25B10 (Figure 2B) bound to LS174T xenografts. mAb B72.3 binding to LS174T sections was heterogeneous (Figure 2A). The sensitivity of LS174T tumor cells to pHAM and BHAMG was determined by measuring [³H]leucine incorporation into cellular proteins after exposure to drugs for 24 h. Comparison of IC₅₀ values showed that BHAMG was 800 times less toxic than pHAM to LS174T cells (Figure 3). The simultaneous addition of β G and BHAMG to tumor cells resulted in a cytotoxic effect equal to pHAM alone, indicating efficient cleavage of the glucuronide functional group of BHAMG.

Tumor Localization of B72.3- β G-PEG. Figure 4 shows that [¹²⁵I]B72.3- β G-PEG preferentially localized in LS174T xenografts. Maximum accumulation of [¹²⁵I]B72.3- β G-PEG in LS174T xenografts ($3.11 \pm 0.8\%$ injected dose/g) was achieved within 48 h after injection. However, the tumor-to-blood ratio at 48 h was only 1.9. The tumor/blood ratio of B72.3- β G-PEG increased to 4.8 at 96 h, but immunoenzyme in tumors decreased about 3-fold from levels achieved at 48 h. [¹²⁵I]H25- β G-PEG did not specifically localize in LS174T xenografts (Figure 4).

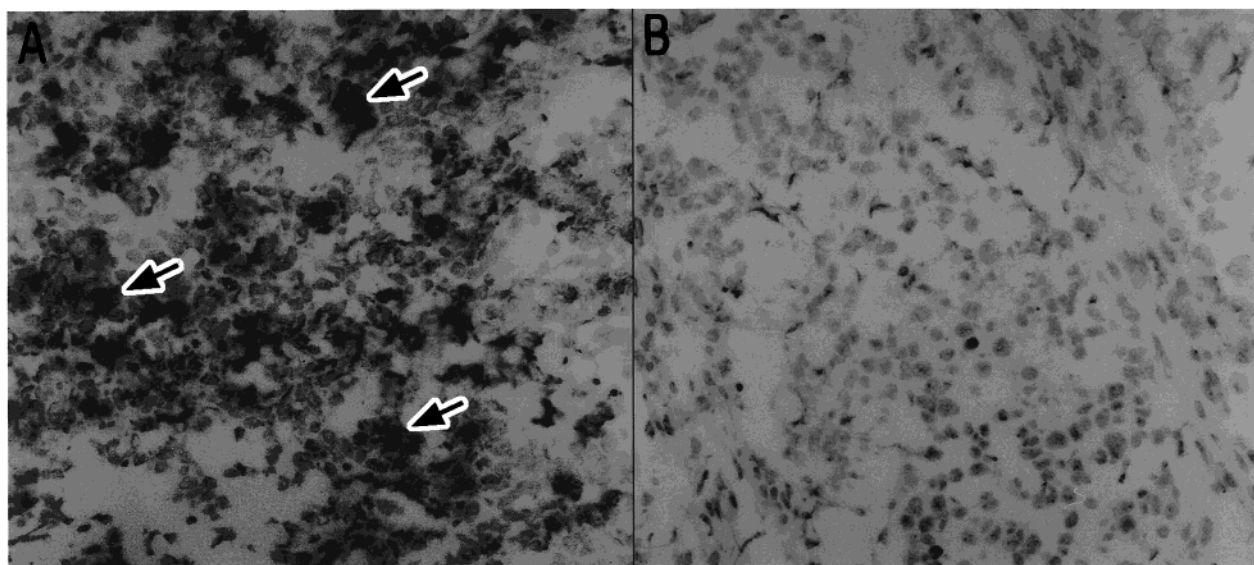


Figure 2. Specificity of B72.3 for LS174T xenografts. Frozen section of LS174T tumors were incubated with mAb B72.3 (A) or H25B10 (B) followed by biotin-labeled goat anti-mouse Ig and streptavidin-conjugated horseradish peroxidase before addition of substrate. Magnification: 200 \times . The sections were counterstained with hematoxylin. Examples of positive immunostaining are indicated with arrows.

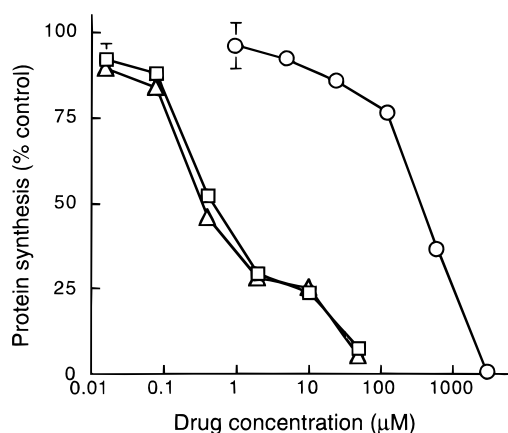


Figure 3. Sensitivity of tumor cells to pHAM and BHAMG. LS174T colon adenocarcinoma cells were exposed to pHAM (\square), BHAMG (\circ), or BHAMG plus 1 $\mu\text{g}/\text{well}$ βG (\triangle) for 24 h, washed with PBS and incubated in fresh medium for 24 h before [^3H]-leucine incorporation was determined. The cellular protein synthesis rate of drug-treated cells is compared to untreated control cells. Bars, SE of triplicate determinations.

In Vivo Clearance of Conjugates. The ability of AGP3, an anti-PEG mAb, to clear B72.3- βG -PEG and H25- βG -PEG from the circulation was examined by i.v. injecting BALB/c mice with 250 μg conjugates followed 48 and 50 h later by two i.v. injections of AGP3. Figure 5 shows that AGP3 reduced the concentration of B72.3- βG -PEG in blood by 33-fold (13.4 to 0.41 $\mu\text{g}/\text{mL}$) and H25- βG -PEG by 65-fold (16.8 to 0.26 $\mu\text{g}/\text{mL}$) in 6 h.

Tumor Localization of B72.3- βG -PEG with AGP3 Clearance. The effect of clearance on tumor localization was determined in BALB/c *nu/nu* mice bearing 100–200 mm^3 LS174T xenografts. Mice were injected with radiolabeled conjugates followed 48 and 50 h later by two i.v. injections of AGP3. After 6 h, tumors, blood, urine, and normal tissues were removed and counted for radioactivity. Figure 6A shows that [^{125}I]B72.3- βG -PEG specifically localized in LS174T tumors. Tumor localization of B72.3- βG -PEG after AGP3 clearance did not significantly ($p > 0.1$) differ from tumor uptake without clearance (2.35 \pm 0.18 versus 2.14 \pm 0.23% injected dose/g). In contrast,

the radioactivity in serum and all tissues except for the colon was lower with AGP3 clearance. This resulted in improved tumor/tissue ratios of B72.3- βG -PEG for most tissues (Table 1). For example, the tumor/blood ratio increased from 3.9 \pm 0.2 to 29.6 \pm 3.1. Although the control conjugate did not specifically accumulate in LS174T xenografts, clearance with AGP3 further decreased the concentration of H25- βG -PEG in the blood and most tissues (Figure 6B). The radioactivity in urine after clearance of both B72.3- βG -PEG and H25- βG -PEG with AGP3 was significantly ($p < 0.005$) greater than without clearance (results not shown). Autoradiographic analysis of urine samples after electrophoresis on SDS-PAGE revealed the presence of low-molecular-weight [^{125}I]-labeled peptides but no intact conjugates (data not shown), indicating that AGP3 clearance resulted in the rapid metabolism of conjugates.

Therapy of LS174T Xenografts. The antitumor activity of BHAMG in combination with B72.3- βG -PEG after clearance of free conjugate with AGP3 was examined. BALB/c *nu/nu* mice bearing 50–100 mm^3 xenografts received two i.v. injections of AGP3 48 and 50 h after i.v. administration of B72.3- βG -PEG or H25- βG -PEG. Mice then received three i.v. injections of BHAMG. Control groups of tumor-bearing mice were treated with BHAMG alone, pHAM alone, or PBS. All mice received two to four rounds of therapy. Figure 7A shows that mean tumor size in mice treated with B72.3- βG -PEG, AGP3, and BHAMG was significantly ($p \leq 0.0005$) smaller than mice receiving other forms of treatment. Control conjugate in combination with AGP3 and BHAMG as well as prodrug or pHAM alone did not significantly ($p > 0.1$) delay tumor growth compared to untreated controls. Table 2 shows that treatment toxicity was minimal with a maximum weight loss of 6% over four rounds of therapy. In contrast, pHAM treatment caused a maximum weight loss of 13% over two rounds of therapy even though it did not provide antitumor activity. Treatment of mice bearing larger LS174T xenografts with a combination of B72.3- βG -PEG, AGP3, and BHAMG also produced significant antitumor activity (Figure 7B).

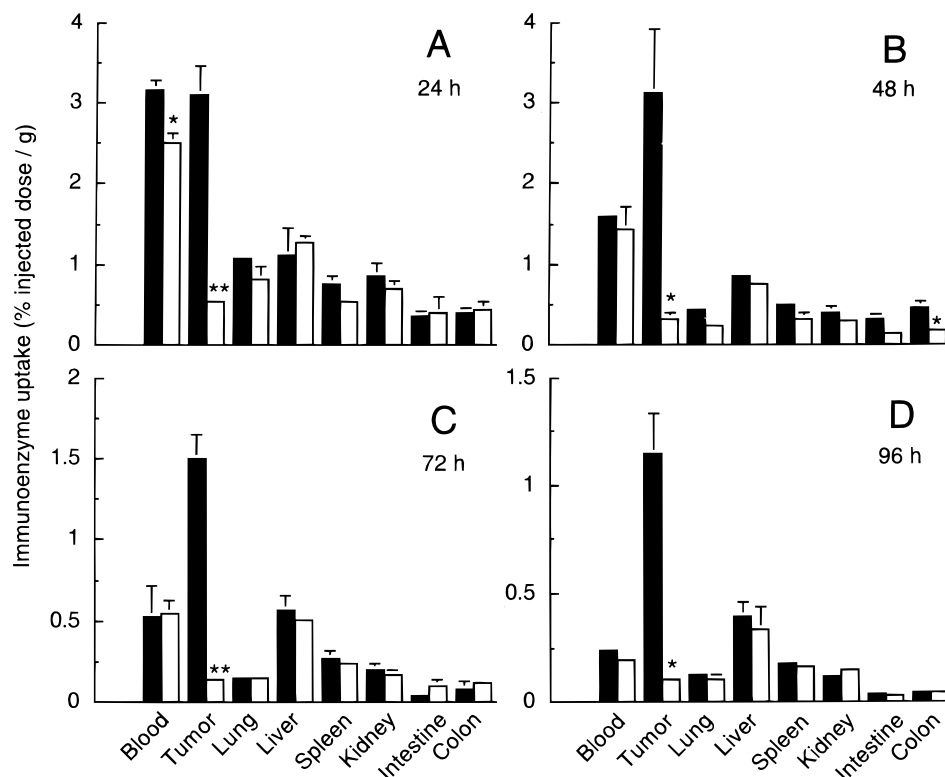


Figure 4. Biodistribution of ^{125}I -labeled immunoconjugates in LS174T tumor-bearing mice. Radioactivity of tumors and tissues was determined (A) 24, (B) 48, (C) 72, and (D) 96 h after nude mice bearing 100–200 mm³ LS174T tumors were i.v. injected with 200 μg (140 μCi) B72.3- βG -PEG (solid bars) or H25- βG -PEG (open bars). Results represent the mean values of three mice. Significant differences between B72.3- βG -PEG and H25- βG -PEG are indicated; (*) $p \leq 0.05$; (**) $p \leq 0.005$. Bars, SE.

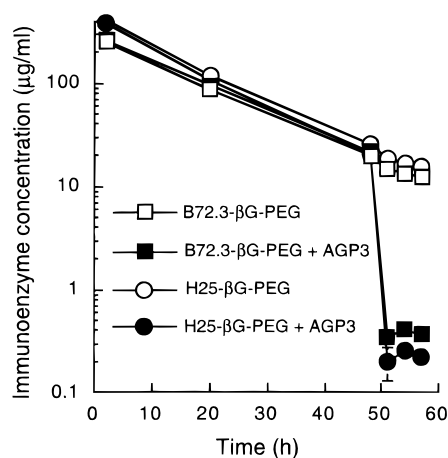


Figure 5. In vivo clearance of immunoconjugates by AGP3 antibody. BALB/c mice were i.v. injected at time 0 with 250 μg B72.3- βG -PEG or H25- βG -PEG. Two i.v. injections of AGP3 (300, 200 μg) or PBS were given at 48 and 50 h. Mean serum concentrations from two to three mice are shown. Bars, SE.

DISCUSSION

mAb B72.3 has been utilized for diagnostic imaging in more than 1000 carcinoma patients with specific tumor localization demonstrated in 70–80% of carcinomas. Cyt-103, ^{111}In -labeled B72.3, is also approved for the detection of extrahepatic intraabdominal metastases from colorectal or ovarian cancer (Divgi, 1996). Preclinical and clinical imaging studies have demonstrated that B72.3 does not internalize after binding to TAG-72 but remains at the tumor site for a number of days following localization (Colcher et al., 1984). Lack of antigen modulation after antibody binding is required to allow efficient activation of BHAMG because this prodrug must be enzymatically

activated outside of tumor cells for maximum cytotoxicity (Cheng et al., 1999a). Although cultured tumor cells express low levels of TAG-72, tumor xenografts display up to a 100-fold increase in antigen expression (Horan Hand et al., 1985). TAG-72 distribution, however, is heterogeneous in both LS174T xenografts (this work) and mucinous adenocarcinoma of the human colon (Schlom, 1986).

βG -PEG was covalently linked to the $\text{F}(\text{ab})_2$ fragment of mAb B72.3 to reduce the size of B72.3- βG -PEG and prevent binding to Fc receptors on hematopoietic cells. We have previously shown that PEG modification of βG allows increased serum half-life and tumor uptake with decreased normal tissues binding (Cheng et al., 1997). In the present study, maximum uptake of [^{125}I]B72.3- βG -PEG in LS174T xenografts was achieved within 48 h. BHAMG could not be administered at this time, however, because serum concentrations of conjugate exceeded 1 $\mu\text{g}/\text{mL}$, the maximum concentration of βG in serum that produced acceptable toxicity from systematically activated prodrug (unpublished results). Without AGP3-mediated clearance, attainment of safe levels of B72.3- βG -PEG in plasma required at least 4 days, at which time the conjugate level in tumor had decreased to 36% of the peak levels at 48 h. Similar or longer intervals are required for other antibody-enzyme conjugates (Bosslet et al., 1994; Svensson et al., 1998), prompting the interest (Sharma et al., 1990; Rogers et al., 1995) in accelerating the clearance of immunoconjugates after maximum tumor uptake has been achieved.

mAb AGP3 has previously been demonstrated to efficiently remove PEG-modified proteins from the circulation (Cheng et al., 1999b). In the present study, we found that AGP3 not only reduced the serum concentration of conjugates by 33–65-fold in 6 h but also accelerated the clearance of immunoconjugate from most normal tissues.

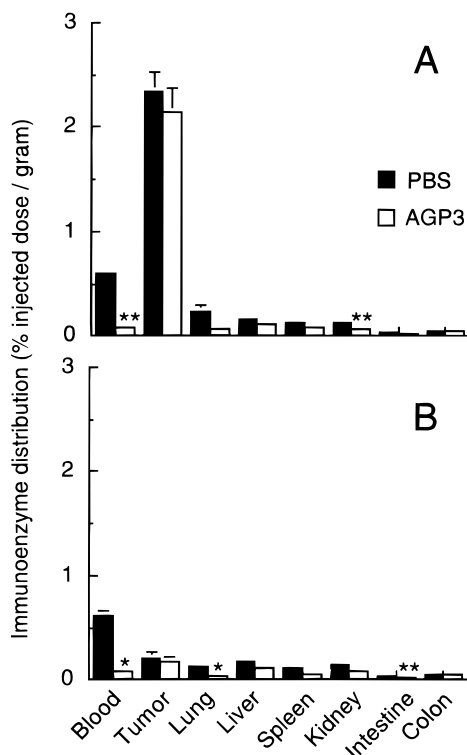


Figure 6. The effect of AGP3 clearance on tumor localization of immunoconjugate. Groups of 6–7 BALB/c *nu/nu* mice bearing 100–200 mm³ LS174T tumor xenografts were i.v. injected at time 0 with 200 μ g (140 μ Ci) [¹²⁵I]B72.3- β G-PEG (A) or [¹²⁵I]-H25- β G-PEG (B). At 48 and 50 h, half the mice were i.v. injected with two fractionated doses of AGP3 (300 and 200 μ g) whereas the other half received PBS. Mice were sacrificed after 6 h and tumors, blood and organs were assayed for radioactivity. Results represent the mean values of 3–4 mice. Significant differences between immunoconjugate uptake with and without AGP3 clearance are indicated; (*) $p \leq 0.05$; (**) $p \leq 0.005$. Bars, SE.

Table 1: Tumor/Tissue Ratio of Conjugates after Clearance with AGP3^a

tissue	tumor/tissue ratio			
	B72.3- β G-PEG		H25- β G-PEG	
	no clearance	AGP3 clearance	no clearance	AGP3 clearance
tumor	1	1	1	1
blood	3.9 \pm 0.2	29.6 \pm 3.1	0.3 \pm 0.1	2.2 \pm 0.8
lung	12.5 \pm 3.3	33.5 \pm 3.0	1.4 \pm 0.4	5.5 \pm 2.1
liver	15.9 \pm 2.9	19.2 \pm 0.8	1.1 \pm 0.2	1.5 \pm 0.6
spleen	19.0 \pm 2.6	27.7 \pm 5.2	1.8 \pm 0.5	3.5 \pm 1.5
kidney	18.1 \pm 1.3	31.0 \pm 2.0	1.4 \pm 0.5	2.2 \pm 0.2
intestine	6.4 \pm 1.7	165 \pm 56	4.8 \pm 1.3	10.1 \pm 2.9
colon	54.3 \pm 7.4	50.2 \pm 8.3	3.6 \pm 0.5	3.1 \pm 0.33
urine	1.1 \pm 0.1	0.6 \pm 0.1	0.1 \pm 0.02	0.03 \pm 0.001

^a Groups of three or four mice bearing 100–200 mm³ solid LS174T tumors were injected with radiolabeled conjugates followed 48 and 50 h later by two i.v. injections of AGP3 or PBS. After 6 h, tumors, blood, urine, and normal tissues were removed and counted in a gamma counter.

Importantly, AGP3 did not significantly reduce tumor accumulation of B72.3- β G-PEG, in contrast to some other clearance systems (Pedley et al., 1989; Sharkey et al., 1992; Kerr et al., 1993). The large size of AGP3 (IgM) may hinder passage of the mAb into the tumor interstitial space, minimizing interactions between localized immunoconjugate and AGP3. The tumor/blood ratios of B72.3- β G-PEG increased from 3.9 to 29.6 at 48 h with AGP3 clearance. This can be contrasted with a tumor/blood ratio of 4.8 at 96 h without clearance. The low tumor/

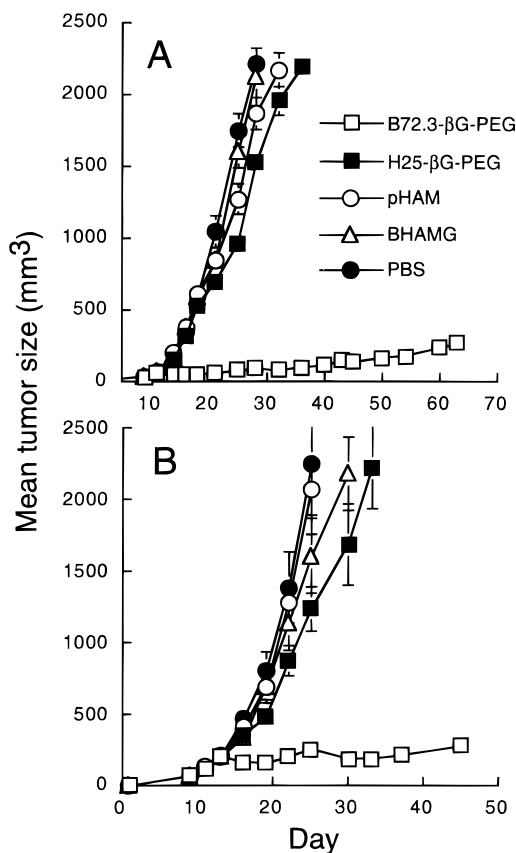


Figure 7. In vivo antitumor activity of prodrug treatment. (A) Groups of 9 BALB/c *nu/nu* mice bearing 50–100 mm³ LS174T tumors were i.v. injected with B72.3- β G-PEG (□) or H25- β G-PEG (■) on day 9 followed by two i.v. injections of AGP3 on day 11. After 6 h, mice were i.v. injected with BHAMG (7.5 mg/kg \times 3). Control groups of tumor-bearing mice were treated with BHAMG (Δ), pHAM (\circ), or PBS (\bullet) alone. Therapy was repeated starting on days 16, 26, and 41. The mean size of tumors in mice sequentially treated with B72.3- β G-PEG, AGP3, and BHAMG was significantly ($p \leq 0.0005$) smaller than control tumors after day 14. B, Groups of eight nude mice bearing 200–250 mm³ LS174T tumors were treated with two round of therapy as above starting on days 11 and 23. The mean size of tumors in mice sequentially treated with B72.3- β G-PEG, AGP3 and BHAMG were significantly ($p \leq 0.005$) smaller than control tumors after day 16. Bars, SE.

Table 2: Toxicity of Therapy^a

	round of therapy			
	1	2	3	4
weight loss %				
PBS	0	0	0	NM
pHAM	6.8	13.0	NM	NM
BHAMG	1.7	4.0	1.7	
B72.3- β G-PEG/AGP3/BHAMG	3.5	5.0	3.8	6.0
H25- β G-PEG/AGP3/BHAMG	3.9	6.6	4.6	NM

^a Groups of 9 BALB/c *nu/nu* mice bearing 50–100 mm³ solid tumors were i.v. injected with PBS, 250 μ g of B72.3- β G-PEG or H25- β G-PEG follow 48 h later by two i.v. injections of AGP3 or PBS. After 6 h, mice were i.v. injected with BHAMG (7.5 mg/kg \times 3). Control groups of mice were treated with BHAMG (7.5 mg/kg \times 3), pHAM (2 mg/kg \times 3) or PBS alone. Mice received 2–4 rounds of therapy. ^b NM, not meaningful due to mouse deaths.

blood ratio without clearance can be attributed to the loss of immunoconjugate from the tumor during the prolonged period required for immunoconjugate to reach safe levels in serum. Clearance of immunoconjugate with AGP3 thus allowed earlier administration of prodrug when immunoconjugate localization at tumor cells was maximal.

Significant antitumor activity with minimal toxicity was demonstrated against LS174T xenografts treated with B72.3- β G-PEG and AGP3 followed by BHAMG administration. The degree of tumor suppression achieved with minimal toxicity by prodrug therapy compares favorably with conventional drugs such as 5-fluorouracil (Blumenthal et al., 1994) and doxorubicin (Meyer et al., 1995) in the LS174T xenograft model. B72.3- β G-PEG appeared to be rapidly catabolized into small peptides that were eliminated in the urine and possibly bile. Soluble IgM immune complexes are primarily removed from the circulation and catabolized by the mononuclear phagocyte system in the liver, spleen, and lungs by receptor-mediated binding of high mannose oligosaccharides exposed upon conformational changes in IgM induced by antigen binding (Day et al., 1980). The low toxicity of BHAMG treatment observed after clearance of B72.3- β G-PEG with AGP3 indicates that cleared conjugate was unavailable for prodrug activation, consistent with rapid degradation of the immunoenzyme after clearance.

mAb B72.3 possesses modest affinity for TAG-72 ($K_a = 2.54 \times 10^9 \text{ M}^{-1}$) (Muraro et al., 1988). High antibody affinity, however, may be critical for the success of immunoconjugate therapy. For example, a β -lactamase immunoenzyme constructed from a high-affinity antibody against melanotransferrin showed greater tumor uptake and produced tumor regressions and cures in mice, whereas a low affinity L6 immunoenzyme was ineffective, even though the expression of L6 antigen was 2-fold higher than that of melanotransferrin (Svensson et al., 1998). Similarly, a high-affinity mAb against ovarian cancer accumulated to higher levels in tumor xenografts compared to a lower affinity mAb (Kievit et al., 1996). Second generation mAbs against TAG-72 with higher affinities [CC49, $K_a = 16.2 \times 10^9 \text{ M}^{-1}$ and CC83, $K_a = 27.7 \times 10^9$ (Muraro et al., 1989)] display improved tumor uptake (O'Boyle et al., 1994) and therapeutic efficacy as radioimmunoconjugates (Schlom et al., 1992) compared with B72.3. Immunoenzymes employing CC49 or CC83 may, therefore, improve the therapeutic efficacy of prodrug treatment.

Most of the enzymes currently under investigation for the targeted activation of anti-neoplastic prodrugs are of microbial origin such as β -lactamase from *Enterobacter cloacae* (Kerr et al., 1995), carboxypeptidase G2 from *Pseudomonas* species (Blakey et al., 1996), β -glucuronidase (Chen et al., 1997), nitroreductase (Anlezark et al., 1995), and penicillin-G amidase (Vrudhula et al., 1993) from *E. coli*, and cytosine deaminase from bakers yeast (Wallace et al., 1994). These enzymes are expected to induce a strong immune response as has been found for carboxypeptidase G2 in a pilot clinical trial (Sharma et al., 1996), thereby limiting the number of times that immunoenzymes can be administered to patients. The utilization of human enzymes to activate antineoplastic prodrugs may not completely prevent this problem since recombinant human proteins can also induce immune responses in patients (Atkins et al., 1986; Gribben et al., 1990). Immunosuppressive drugs such as cyclosporin A, cyclophosphamide, and deoxyspergualin can decrease or delay the immune response against antibodies (Leder-mann et al., 1991), immunotoxins (Pai et al., 1990) and antibody-enzyme conjugates (Sharma et al., 1996). Immunosuppression, in addition to producing toxicity in some patients (Sharma et al., 1996), may hinder the development of antitumor immunity generated by prodrug therapy (Chen et al., 1997).

Immune responses against proteins are attenuated by attachment of PEG (Abuchowski et al., 1977). PEG, in contrast to immunosuppressive drugs, is not toxic and does not affect systemic immunity. PEG modification has been shown to reduce the immunogenicity of enzymes (Abuchowski et al., 1977), antibodies (Kitamura et al., 1991), toxins (Wang et al., 1993), and recombinant human proteins (Katre, 1990). PEG modification of bacterial enzymes may allow repeated administration of immunoenzymes for ADEPT without the need to employ toxic immunosuppressive drugs. PEG-modified enzymes of the proper molecular size can also preferentially accumulate in tumors and are under investigation for tumor selective prodrug activation (Bagshawe et al., 1999). AGP3 binds to the backbone of PEG (Cheng et al., 1997 and unpublished results) independent of the linker or protein employed. AGP3 should therefore be generally applicable to the accelerated clearance of PEG-modified immunoenzymes, radioimmunoconjugates and imaging agents as well as for the analysis of PEG-modified proteins.

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LITERATURE CITED

- Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252, 3582–3586.
- Anlezark, G. M., Melton, R. G., Sherwood, R. F., Wilson, W. R., Denny, W. A., Palmer, B. D., Knox, R. J., Friedlos, F., and Williams, A. (1995) Bioactivation of dinitrobenzamide mustards by an *E. coli* B nitroreductase. *Biochem. Pharmacol.* 50, 609–618.
- Atkins, M. B., Gould, J. A., Allegretta, M., Li, J. J., Dempsey, R. A., Rudders, R. A., Parkinson, D. R., Reichlin, S., and Mier, J. W. (1986) Phase I evaluation of recombinant interleukin-2 in patients with advanced malignant disease. *J. Clin. Oncol.* 4, 1380–1391.
- Bagshawe, K. D., Sharma, S. K., Know, R., and Amooquaye, E. (1999) Studies with polymer enzyme conjugates and AMIRACS. *Advances in the Application of Monoclonal Antibodies in Clinical Oncology*, Vol. 28, Samos, Greece.
- Bagshawe, K. D., Springer, C. J., Searle, F., Antoniwi, P., Sharma, S. K., Melton, R. G., and Sherwood, R. F. (1988) A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* 58, 700–703.
- Blakey, D. C., Burke, P. J., Davies, D. H., Dowell, R. I., East, S. J., Eckersley, K. P., Fitton, J. E., McDaid, J., Melton, R. G., Niculescu Duvaz, I. A., Pinder, P. E., Sharma, S. K., Wright, A. F., and Springer, C. J. (1996) ZD2767, an improved system for antibody-directed enzyme prodrug therapy that results in tumor regressions in colorectal tumor xenografts. *Cancer Res.* 56, 3287–3292.
- Blumenthal, R. D., Sharkey, R. M., Natale, A. M., Kashi, R., Wong, G., and Goldenberg, D. M. (1994) Comparison of equitoxic radioimmunotherapy and chemotherapy in the treatment of human colonic cancer xenografts. *Cancer Res.* 54, 142–151.
- Bosslet, K., Czech, J., and Hoffmann, D. (1994) Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res.* 54, 2151–2159.
- Chen, B. M., Chan, L. Y., Wang, S. M., Wu, M. F., Chern, J. W., and Roffler, S. R. (1997) Cure of malignant ascites and generation of protective immunity by monoclonal antibody-targeted activation of a glucuronide prodrug in rats. *Int. J. Cancer* 73, 392–402.
- Cheng, T. L., Chen, B. M., Chan, L. Y., Wu, P. Y., Chern, J. W., and Roffler, S. R. (1997) Poly(ethylene glycol) modification

- of beta-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs. *Cancer Immunol. Immunother.* **44**, 305–315.
- Cheng, T. L., Chou, W. C., Chen, B. M., Chern, J. W., and Roffler, S. R. (1999a) Characterization of an antineoplastic glucuronide prodrug. *Biochem. Pharmacol.* **58**, 325–328.
- Cheng, T. L., Wu, P. Y., Wu, M. F., Chern, J. W., and Roffler, S. R. (1999b) Accelerated clearance of polyethylene glycol-modified proteins by anti-polyethylene glycol IgM. *Bioconjugate Chem.* **10**, 520–528.
- Colcher, D., Keenan, A. M., Larson, S. M., and Schlom, J. (1984) Prolonged binding of a radiolabeled monoclonal antibody (B72.3) used for the in situ radioimmunoassay of human colon carcinoma xenografts. *Cancer Res.* **44**, 5744–5751.
- Day, J. F., Thornburg, R. W., Thorpe, S. R., and Baynes, J. W. (1980) Carbohydrate-mediated clearance of antibody-antigen complexes from the circulation. The role of high mannose oligosaccharides in the hepatic uptake of IgM-antigen complexes. *J. Biol. Chem.* **255**, 2360–2365.
- Delgado, C., Francis, G. E., and Fisher, D. (1992) The uses and properties of PEG-linked proteins. *Crit. Rev. Ther. Drug Carrier Sys.* **9**, 249–304.
- Delgado, C., Pedley, R. B., Herraiz, A., Boden, R., Boden, J. A., Keep, P. A., Chester, K. A., Fisher, D., Begent, R. H., and Francis, G. E. (1996) Enhanced tumour specificity of an anti-carcinoembryonic antigen Fab' fragment by poly(ethylene glycol) (PEG) modification. *Br. J. Cancer* **73**, 175–182.
- Divgi, C. R. (1996) Status of radiolabeled monoclonal antibodies for diagnosis and therapy of cancer. *Oncology (Huntingt)* **10**, 939–953.
- Eccles, S. A., Court, W. J., Box, G. A., Dean, C. J., Melton, R. G., and Springer, C. J. (1994) Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. *Cancer Res.* **54**, 5171–5177.
- Ey, P. L., Prowse, S. J., and Jenkin, C. R. (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry* **15**, 429–436.
- Gribben, J. G., Devereux, S., Thomas, N. S., Keim, M., Jones, H. M., Goldstone, A. H., and Linch, D. C. (1990) Development of antibodies to unprotected glycosylation sites on recombinant human GM-CSF. *Lancet* **335**, 434–437.
- Haisma, H. J., Van Muijen, M., Scheffer, G., Scheper, R. J., Pinedo, H. M., and Boven, E. (1995) A monoclonal antibody against human beta-glucuronidase for application in antibody-directed enzyme prodrug therapy. *Hybridoma* **14**, 377–382.
- Horan Hand, P., Colcher, D., Salomon, D., Ridge, J., Noguchi, P., and Schlom, J. (1985) Influence of spatial configuration of carcinoma cell populations on the expression of a tumor-associated glycoprotein. *Cancer Res.* **45**, 833–840.
- Johnson, V. G., Schlom, J., Paterson, A. J., Bennett, J., Magnani, J. L., and Colcher, D. (1986) Analysis of a human tumor-associated glycoprotein (TAG-72) identified by monoclonal antibody B72.3. *Cancer Res.* **46**, 850–857.
- Katre, N. V. (1990) Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol. *J. Immunol.* **144**, 209–213.
- Kerr, D. E., Garrigues, U. S., Wallace, P. M., Hellstrom, K. E., Hellstrom, I., and Senter, P. D. (1993) Application of monoclonal antibodies against cytosine deaminase for the in vivo clearance of a cytosine deaminase immunoconjugate. *Bioconjugate Chem.* **4**, 353–357.
- Kerr, D. E., Schreiber, G. J., Vrduhula, V. M., Svensson, H. P., Hellstrom, I., Hellstrom, K. E., and Senter, P. D. (1995) Regressions and cures of melanoma xenografts following treatment with monoclonal antibody beta-lactamase conjugates in combination with anticancer prodrugs. *Cancer Res.* **55**, 3558–3563.
- Kievit, E., Pinedo, H. M., Schluper, H. M., Haisma, H. J., and Boven, E. (1996) Comparison of monoclonal antibodies 17-1A and 323/A3: the influence of the affinity on tumour uptake and efficacy of radioimmunotherapy in human ovarian cancer xenografts. *Br. J. Cancer* **73**, 457–464.
- King, D. J., Turner, A., Farnsworth, A. P., Adair, J. R., Owens, R. J., Pedley, R. B., Baldock, D., Proudfoot, K. A., Lawson, A. D., and Beeley, N. R., et al. (1994) Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.* **54**, 6176–6185.
- Kitamura, K., Takahashi, T., Yamaguchi, T., Noguchi, A., Takashina, K., Tsurumi, H., Inagake, M., Toyokuni, T., and Hakomori, S. (1991) Chemical engineering of the monoclonal antibody A7 by polyethylene glycol for targeting cancer chemotherapy. *Cancer Res.* **51**, 4310–4315.
- Ledermann, J. A., Begent, R. H., Massof, C., Kelly, A. M., Adam, T., and Bagshawe, K. D. (1991) A phase-I study of repeated therapy with radiolabelled antibody to carcinoembryonic antigen using intermittent or continuous administration of cyclosporin A to suppress the immune response. *Int. J. Cancer* **47**, 659–664.
- Meyer, D. L., Law, K. L., Payne, J. K., Mikolajczyk, S. D., Zarrinmayeh, H., Jungheim, L. N., Kling, J. K., Shepherd, T. A., and Starling, J. J. (1995) Site-specific prodrug activation by antibody-beta-lactamase conjugates: preclinical investigation of the efficacy and toxicity of doxorubicin delivered by antibody directed catalysis. *Bioconjugate Chem.* **6**, 440–446.
- Milenic, D. E., Esteban, J. M., and Colcher, D. (1989) Comparison of methods for the generation of immunoreactive fragments of a monoclonal antibody (B72.3) reactive with human carcinomas. *J. Immunol. Methods* **120**, 71–83.
- Muraro, R., Kuroki, M., Wunderlich, D., Poole, D. J., Colcher, D., Thor, A., Greiner, J. W., Simpson, J. F., Molinolo, A., and Noguchi, P., et al. (1988) Generation and characterization of B72.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein 72 antigen. *Cancer Res.* **48**, 4588–4596.
- Muraro, R., Nuti, M., Natali, P. G., Bigotti, A., Simpson, J. F., Primus, F. J., Colcher, D., Greiner, J. W., and Schlom, J. (1989) A monoclonal antibody (D612) with selective reactivity for malignant and normal gastro-intestinal epithelium. *Int. J. Cancer* **43**, 598–607.
- O'Boyle, K. P., Goya, V., Zuckier, L. S., Chun, S., and Bhargava, K. (1994) Expression of human tumor mucin-associated carbohydrate epitopes, including sialylated Tn, and localization of murine monoclonal antibodies CC49 and B72.3 in a syngeneic rat colon carcinoma model. *J. Immunother. Emphasis Tumor Immunol.* **16**, 251–261.
- Paganelli, G., Stella, M., De Nardi, P., Magnani, P., Zito, F., Siccardi, A. G., Di Carlo, V., and Fazio, F. (1991) A new method for faster blood clearance in radioimmuno-guided surgery. *J. Nucl. Biol. Med.* **35**, 88–89.
- Pai, L. H., FitzGerald, D. J., Tepper, M., Schacter, B., Spitalny, G., and Pastan, I. (1990) Inhibition of antibody response to Pseudomonas exotoxin and an immunotoxin containing Pseudomonas exotoxin by 15-deoxyspergualin in mice. *Cancer Res.* **50**, 7750–7753.
- Pedley, R. B., Boden, J. A., Boden, R., Begent, R. H., Turner, A., Haines, A. M., and King, D. J. (1994) The potential for enhanced tumour localisation by poly(ethylene glycol) modification of anti-CEA antibody. *Br. J. Cancer* **70**, 1126–1130.
- Pedley, R. B., Dale, R., Boden, J. A., Begent, R. H., Keep, P. A., and Green, A. J. (1989) The effect of second antibody clearance on the distribution and dosimetry of radiolabelled anti-CEA antibody in a human colonic tumor xenograft model. *Int. J. Cancer* **43**, 713–718.
- Roffler, S. R., Wang, S. M., Chern, J. W., Yeh, M. Y., and Tung, E. (1991) Anti-neoplastic glucuronide prodrug treatment of human tumor cells targeted with a monoclonal antibody-enzyme conjugate. *Biochem. Pharmacol.* **42**, 2062–2065.
- Rogers, G. T., Burke, P. J., Sharma, S. K., Koodie, R., and Boden, J. A. (1995) Plasma clearance of an antibody-enzyme conjugate in ADEPT by monoclonal anti-enzyme: its effect on prodrug activation in vivo. *Br. J. Cancer* **72**, 1357–1363.
- Schlom, J. (1986) Basic principles and applications of monoclonal antibodies in the management of carcinomas: the Richard and Hinda Rosenthal Foundation award lecture. *Cancer Res.* **46**, 3225–3238.
- Schlom, J., Eggensperger, D., Colcher, D., Molinolo, A., Houchens, D., Miller, L. S., Hinkle, G., and Siler, K. (1992) Therapeutic advantage of high-affinity anticarcinoma radioimmunoconjugates. *Cancer Res.* **52**, 1067–1072.
- Senter, P. D., Saulnier, M. G., Schreiber, G. J., Hirschberg, D. L., Brown, J. P., Hellstrom, I., and Hellstrom, K. E. (1988)

- Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4842–4846.
- Sharkey, R. M., Boerman, O. C., Natale, A., Pawlyk, D., Monestier, M., Losman, M. J., and Goldenberg, D. M. (1992) Enhanced clearance of radiolabeled murine monoclonal antibody by a syngeneic anti-idiotypic antibody in tumor-bearing nude mice. *Int. J. Cancer* **51**, 266–273.
- Sharma, S. K., Bagshawe, K. D., Burke, P. J., Boden, R. W., and Rogers, G. T. (1990) Inactivation and clearance of an anti-CEA carboxypeptidase G2 conjugate in blood after localisation in a xenograft model. *Br. J. Cancer* **61**, 659–662.
- Sharma, S. K., Bagshawe, K. D., Melton, R. G., and Begent, R. H. (1996) Effect of cyclosporine on immunogenicity of a bacterial enzyme carboxypeptidase G2 in ADEPT. *Transplant Proc.* **28**, 3154–3155.
- Siemers, N. O., Kerr, D. E., Yarnold, S., Stebbins, M. R., Vrudhula, V. M., Hellstrom, I., Hellstrom, K. E., and Senter, P. D. (1997) Construction, expression, and activities of L49-sFv-beta-lactamase, a single-chain antibody fusion protein for anticancer prodrug activation. *Bioconjugate Chem.* **8**, 510–519.
- Stewart, J. S., Sivolapenko, G. B., Hird, V., Davies, K. A., Walport, M., Ritter, M. A., and Epenetos, A. A. (1990) Clearance of ¹³¹I-labeled murine monoclonal antibody from patients' blood by intravenous human anti-murine immunoglobulin antibody. *Cancer Res.* **50**, 563–567.
- Svensson, H. P., Frank, I. S., Berry, K. K., and Senter, P. D. (1998) Therapeutic effects of monoclonal antibody-beta-lactamase conjugates in combination with a nitrogen mustard anticancer prodrug in models of human renal cell carcinoma. *J. Med. Chem.* **41**, 1507–1512.
- Tennvall, J., Garkavij, M., Chen, J., Sjogren, H. O., and Strand, S. E. (1997) Improving tumor-to-normal-tissue ratios of antibodies by extracorporeal immunoabsorption based on the avidin–biotin concept: development of a new treatment strategy applied to monoclonal antibodies murine L6 and chimeric BR96. *Cancer* **80**, 2411–2418.
- Thor, A., Ohuchi, N., Szpak, C. A., Johnston, W. W., and Schlom, J. (1986) Distribution of oncofetal antigen tumor-associated glycoprotein-72 defined by monoclonal antibody B72.3. *Cancer Res.* **46**, 3118–3124.
- Ullen, A., Sandstrom, P., Ahlstrom, K. R., Sundstrom, B., Nilsson, B., Arlestig, L., and Stigbrand, T. (1995) Use of anticytokeratin monoclonal anti-idiotypic antibodies to improve tumor: nontumor ratio in experimental radioimmunolocalization. *Cancer Res.* **55**, 5868s–5873s.
- Vrudhula, V. M., Senter, P. D., Fischer, K. J., and Wallace, P. M. (1993) Prodrugs of doxorubicin and melphalan and their activation by a monoclonal antibody-penicillin-G amidase conjugate. *J. Med. Chem.* **36**, 919–923.
- Wallace, P. M., MacMaster, J. F., Smith, V. F., Kerr, D. E., Senter, P. D., and Cosand, W. L. (1994) Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res.* **54**, 2719–2723.
- Wang, Q. C., Pai, L. H., Debinski, W., FitzGerald, D. J., and Pastan, I. (1993) Polyethylene glycol-modified chimeric toxin composed of transforming growth factor alpha and Pseudomonas exotoxin. *Cancer Res.* **53**, 4588–4594.
- Wang, S. M., Chern, J. W., Yeh, M. Y., Ng, J. C., Tung, E., and Roffler, S. R. (1992) Specific activation of glucuronide prodrugs by antibody-targeted enzyme conjugates for cancer therapy. *Cancer Res.* **52**, 4484–4491.

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