Accelerated Clearance of Polyethylene Glycol-Modified Proteins by Anti-Polyethylene Glycol IgM

Tian-Lu Cheng,[†,‡] Pin-Yi Wu,[‡] Ming-Fang Wu,[§] Ji-Wang Chern,[∥] and Steve R. Roffler*[,‡]

Institute of Biomedical Sciences, Academia Sinica; Graduate Institute of Life Sciences, National Defense Medical Center; College of Medicine, National Taiwan University; School of Pharmacy, National Taiwan University, Taipei, Taiwan. Received December 2, 1998; Revised Manuscript Received March 3, 1999

Tumor therapy by the preferential activation of a prodrug at tumor cells targeted with an antibody−enzyme conjugate may allow improved treatment efficacy with reduced side effects. We examined antibody-mediated clearance of poly(ethylene glycol)-modified β-glucuronidase (βG−sPEG) as a method to reduce serum concentrations of enzyme and minimize systemic prodrug activation. Enzyme-linked immunosorbent assay and immunoblot analysis of two monoclonal antibodies generated by immunization of BALB/c mice with an antibody−βG−sPEG conjugate showed that mAb 1E8 (IgG1) bound βG and βG−sPEG whereas mAb AGP3 (IgM) bound poly(ethylene glycol). Neither antibody affected the βG activity. mAb 1E8 and AGP3 were modified with 36 and 208 galactose residues (1E8−36G and AGP3−208G) with retention of 72 and 48% antigen-binding activity, respectively, to target immune complexes to the asialoglycoprotein receptor on liver cells. mAb 1E8 and AGP3 cleared βG−PEG from the circulation of mice as effectively as 1E8−36G and AGP3−208G, respectively. mAb AGP3, however, cleared βG−sPEG more completely and rapidly than 1E8, reducing the serum concentration of βG−sPEG by 38-fold in 8 h. AGP3 also reduced the concentration of an antibody−βG−sPEG conjugate in blood by 280-fold in 2 h and 940-fold in 24 h. AGP3-mediated clearance did not produce obvious damage to liver, spleen, or kidney tissues. In addition, AGP3 clearance of βG−sPEG before administration of BHAMG, a glucuronide prodrug of *p*-hydroxyaniline mustard, prevented toxicity associated with systemic activation of the prodrug based on mouse weight and blood cell numbers. AGP3 should be generally useful for accelerating the clearance of PEG-modified proteins as well as for improving the tumor/blood ratios of antibody−βG−PEG conjugates for glucuronide prodrug therapy of cancer.

INTRODUCTION

Drugs, toxins, and isotopes can be selectively delivered to tumors by creating conjugates with monoclonal antibodies (mAb)[1] that preferentially bind tumor-associated antigens. While high serum concentrations of immunoconjugates can increase accumulation and provide more uniform distribution in tumors (Thomas et al., 1989; Fujimori et al., 1990), low serum concentrations of cytotoxic conjugates are preferable to minimize systemic toxicity. High tumor/blood ratios are also desirable for noncytotoxic immunoconjugates such as antibody−enzyme conjugates employed for prodrug activation to minimize systemic prodrug activation.

Tumor/blood ratios of immunoconjugates can be increased by employing an agent that preferentially clears conjugate from the circulation. Avidin has been used to accelerate the clearance of biotinylated antibodies (Klibanov et al., 1988; Stella et al., 1994; Kobayashi et al., 1995) and improve the localization in tumors (Yao et al., 1995; Zhang et al., 1997). Clearance of biotinylated immunoconjugates, however, can be adversely impacted by the high concentrations of endogenous biotin present in serum (Guilarte, 1985) as well as by cleavage of biotin by biotinidase in serum (Wolf et al., 1990). Alternatively, antibodies against primary antibodies (Goldenberg et al. 1987; Sharkey et al., 19984) and antibody−enzyme conjugates (Kerr et al., 1993; Wallace et al., 1994) have also been employed to promote the clearance and tumor localization of immunoconjugates.

In the present study, we were interested in developing a clearance system for PEG-modified conjugates for use in cancer therapy by antibody-directed enzyme activation of glucuronide prodrugs. β-Glucuronidase targeted to tumor cells can activate the glucuronide prodrug (BHAMG) of *p*-hydroxy aniline mustard (pHAM) in vitro (Wang et al., 1992) and cure advanced hepatoma ascites in a rat model (Chen et al., 1997). Antibody−βG conjugates injected intravenously, however, are rapidly cleared from

* To whom correspondence should be addressed. Phone: +886 22 652 3079. Fax: +886 22 782 9142. E-mail: sroff@ibms.sinica.edu.tw.

† Graduate Institute of Life Sciences.

‡ Institute of Biomedical Sciences.

§ College of Medicine.

∥ School of Pharmacy.

[1] Abbreviations: 1E8-*x*G, mAb 1E8 containing *x* galactose moieties per antibody; 5A8-βG−PEG, conjugate formed between mAb 5A8 and βG−sPEG; AGP3-*x*G, mAb AGP3 containing *x* galactose moieties per antibody; βG, *Escherichia coli* β-glucuronidase; βG−sPEG, β-glucuronidase modified with sPEG; βG-tPEG, β-glucuronidase modified with tPEG; BHAMG, glucuronide prodrug of pHAM; BSA, bovine serum albumin; BSA−sPEG, bovine serum albumin modified with sPEG; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween-20; PEG, monomethoxy poly(ethylene glycol); pHAM, *p*-hydroxy aniline mustard; PLT, platelets; RBC, red blood cells; RH1−βG−PEG, conjugate formed between mAb RH1 and βG−sPEG; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate; sPEG, methoxypoly(ethylene glycol) succinimidyl succinate; tPEG, methoxypoly(ethylene glycol) tresylate; WBC, white blood cells.

10.1021/bc980143z CCC: $18.00
Published on Web 04/17/1999

the circulation before significant tumor accumulation can occur (Cheng et al., 1997). Modification of βG with PEG can extend the half-life of antibody−βG conjugates, decrease normal tissue uptake, and increase the localization of conjugate at solid tumors in nude mice (Cheng et al., 1997). Although the extended half-life of PEG-modified βG conjugates is desirable for improved tumor uptake, at least 5 days were required for the serum concentration of conjugate to reach safe levels before prodrug could be administered (Cheng et al., 1997). Accelerated clearance of conjugates from the circulation may allow earlier prodrug administration when the greatest amount of conjugate is present at tumor cells. In the present study, we generated mAbs against βG and PEG and examined their effect on the clearance of βG−sPEG conjugates. The effect of incorporating galactose residues in the antibodies on the clearance of βG−sPEG conjugates was also examined because galactose can accelerate the removal of proteins from the circulation (Thornburg et al., 1980) by the hepatic asialoglycoprotein receptor (Ong et al., 1991). Rapid clearance of an antibody−carboxypeptidase G2 conjugate by a galactose-modified antibody (Sharma et al., 1990, 1994) allowed earlier administration of a prodrug with reduced toxicity (Rogers et al., 1995). In addition, galactose-modified mAb has also been employed in a clinical trial to remove residual antibody−carboxypeptidase G2 conjugate from the circulation before prodrug administration (Martin et al., 1997). We show that an IgM antibody against PEG can rapidly clear PEG-containing conjugates from the circulation without toxicity and that galactose modification of the IgM is unnecessary.

EXPERIMENTAL PROCEDURES

BSA (Fraction V), sPEG, tPEG, cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside, trinitrobenzenesulfonic acid, fluorescamine, and *p*-nitrophenyl β-D-glucuronide were purchased from Sigma (St. Louis, MO). Sepharose S-200 HR, Sepharose S-300 HR and Sephadex G-25 gels were from Pharmacia Biotech Asia Pacific Ltd. (Taiwan). pHAM and BHAMG were synthesized as described (Roffler et al., 1991). SMCC was from Pierce Chemical Company, Rockford, IL. Second antibodies were purchased from Organon (Durham, NC). FO myeloma cells were purchased from ATCC, Manassas, VA. BALB/c mice were obtained from the animal room of the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan.

PEG-Modification of βG. Recombinant βG was produced and purified as described (Cheng et al., 1997). Twenty milligrams of sPEG per milligram of βG (2 mg/mL) was mixed in coupling buffer (deoxygenated PBS containing 1 mM EDTA, pH 8.0) at room temperature for 2 h before one-tenth the volume of a saturated solution of glycine in coupling buffer was added to stop the reaction. Unreacted PEG was removed by extensive dialysis against PBS or by gel filtration on a 2.5 × 100 cm Sepharose S-200 HR column eluted with PBS at 15 mL/h. Relevant fractions were pooled and concentrated by ultrafiltration to 1.5−2.0 mg/mL for storage at −80 °C. BSA was modified with sPEG in a similar manner. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) with BSA employed as the reference protein. The BCA assay was not affected by covalently coupled PEG after removal of free PEG. In some experiments, βG in coupling buffer was modified with 2 mg of sPEG or with 2 or 20 mg of tPEG/mg of βG.

Conjugation of antibodies to βG−sPEG. mAb RH1 (Roffler et al., 1994) and 5A8 (Maloney et al., 1985) were passed through a 2.6 × 30 cm Sephadex G-25 column equilibrated with coupling buffer and concentrated by ultrafiltration to 3 mg/mL. A 4-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 × 30 cm Sephadex G-25 column equilibrated with coupling buffer. The average number of maleimido groups introduced into antibodies (1−1.5) was assessed as described (Ishikawa et al., 1987). βG−sPEG, prepared as described above, was partially reduced by adding dithiothreitol to a final concentration of 20 mM for 1 h at 37 °C. βG−sPEG was then desalted on a 2.6 × 30 cm Sephadex G-50 column equilibrated with coupling buffer and concentrated to 2 mg/mL. Freshly derivatized antibody and βG−sPEG were immediately mixed at equal molar ratios, concentrated to 2 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 about 5 mg/mL. Conjugates were purified by gel filtration on a 2.6 × 100 cm Sepharose 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 1.5−2.0 mg/mL for storage at −80 °C. The yield of conjugates averaged around 35% based on the weight of purified conjugate divided by the total starting weights of antibody and enzyme.

Generation of Monoclonal Antibodies against βG and βG−PEG. Female BALB/c mice were i.v. injected with 200 μg of RH1−βG−PEG in PBS on day 1 and i.p. injected with 100 μg of RH1−βG−PEG on days 7, 14, 21, and 28. Mice received a final i.v. injection of 200 μg of RH1−βG−PEG on day 54. Hybridomas were generated by fusing spleen cells with FO myeloma cells 3 days later (Yeh et al., 1979). Hybridomas were screened by ELISA in 96-well microtiter plates coated with 1 μg/well βG or βG−PEG. Selected hybridomas were cloned 3 times by limiting dilution in 96-well microtiter plates containing thymocyte feeder cells in HT medium supplemented with 15% fetal calf serum. The isotypes and subclasses of mAbs were determined with the Mouse MonoAb-ID kit according to the manufacturer's instructions (Zymed laboratories, South San Francisco, CA). The apparent affinity constants (avidity) of antibodies for βG and βG−sPEG were measured as described (Beatty et al., 1987).

Antibody ELISA. Microtiter plates were coated with 1 μg/well βG, βG−sPEG, BSA, or BSA−sPEG in PBS and blocked with 2% skim milk. Serial dilutions of 1E8 and AGP3 were added to the plates for 1 h at 37 °C. The plates were washed 3 times with PBS-T and 3 times with PBS before horseradish peroxidase conjugated second antibody was added for 1 h at 37 °C. Plates were washed and bound peroxidase activity was measured by adding 100 μL/well ABTS solution [0.4 mg/mL 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, 100 mM phosphate-citrate, pH 4.0] for 15 min at room temperature. Absorbance (405 nm) of wells was measured in a microplate reader (Molecular Device, Menlo Park, CA).

Immunoblotting of PEG-Modified Proteins. Samples were electrophoresed in sodium-dodecyl polyacrylamide gels under reducing conditions before overnight transfer to nitrocellulose paper by passive diffusion in blotting buffer (50 mM NaCl, 2 mM EDTA, 0.5 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 7.5). Blots were blocked with 5% skim milk and incubated for 1 h at 37 °C with 1E8 or AGP3 ascites diluted 1:2000 in PBS containing

0.05% BSA. Blots were washed 3 times with PBS-T and twice with PBS before incubation with horseradish peroxidase conjugated second antibody for 30 min at 37 °C. Blots were washed and bands were visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL).

Galactose-modification of antibodies. Cyanomethyl-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside was reacted with 0.1 M sodium methoxide in dry methanol for 48 h (Marshall et al., 1995). The methanol was removed by evaporation under vacuum and antibodies (5 mg/mL in pH 8.5 borate buffer) were directly added and allowed to react for 2 h. Different amounts of galactose were incorporated into antibodies by varying the ratio of the reactants. The number of galactose groups introduced into antibodies was estimated by the reduction of trinitrobenzesulfonic acid reactive amine groups after galactose modification (Habeeb, 1966) using a molar extinction coefficient (ϵ^{335}) of 1.31×10^4 for 1E8 and 1.38×10^4 for AGP3. Galactose groups were also estimated by measuring amine groups before and after galactose modification with fluorescamine (Stocks et al., 1986).

Serum Half-Lives of Antibodies. BALB/c mice were i.v. injected with 200 μg of unmodified or galactose-modified antibody. Serum was isolated from blood samples that were periodically removed from the tail vein of mice. The concentrations of 1E8 or AGP3 were determined in duplicate samples by ELISA in microtiter plates coated with βG or βG−sPEG, respectively, by comparison to standard curves generated with known concentrations of the corresponding unmodified or galactose-modified antibody.

Clearance of βG−PEG and 5A8-βG−PEG from Serum. Groups of 3 BALB/c mice were i.v. injected with βG−sPEG or 5A8−βG−PEG at time zero. Blood samples were periodically removed before i.v. injection of unmodified or galactose-modified 1E8 or AGP3 at 24 h. Additional blood samples were taken at subsequent times and the βG 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 βG−sPEG or 5A8−βG−sPEG. For meaningful comparison of clearance results, the measured concentrations of βG−sPEG in mice injected with clearing antibodies were normalized to the mean concentration of βG−sPEG in control (uncleared) mice just before clearance by

$$N_j = (C_{\mathrm{mi}}/C_{\mathrm{i}})C_j$$

where N_j is the normalized serum concentration of βG−sPEG in an experimental mouse at time j, C_{mi} is the mean serum concentration of βG−sPEG in the control group of mice at the last time point before clearance, C_{i} is the serum concentration of βG−sPEG in an experimental mouse at the last time point before clearance, and C_j is the serum concentration of βG−sPEG in an experimental mouse at time j.

Toxicity of Clearance. The number of WBC, RBC, and PLT in groups of 6 BALB/c mice were measured as described (Chen et al., 1997) 1 day before i.v. injection of 50 μg of βG−PEG or PBS. Mice were i.v. injected 24 h later with 300 μg of AGP3 or AGP3−208G or two fractionated 150 μg doses of AGP3−208G separated by 4 h. After allowing 6 h for clearance, mice were i.v. injected with two 7.5 mg/kg doses of BHAMG in PBS. On day 8, the number of WBC, RBC, and PLT in serum samples were determined. On days 3 and 10, the spleen, liver, and kidney were removed from one mouse in each group and fixed in 10% formalin. Ethanol dehydrated samples were embedded in paraffin and cut into 3 μm sections. Sections were then dewaxed with xylene, stained with hematoxylin/eosin, and dehydrated before observation under a light microscope. The weight of mice was followed for 18 days.

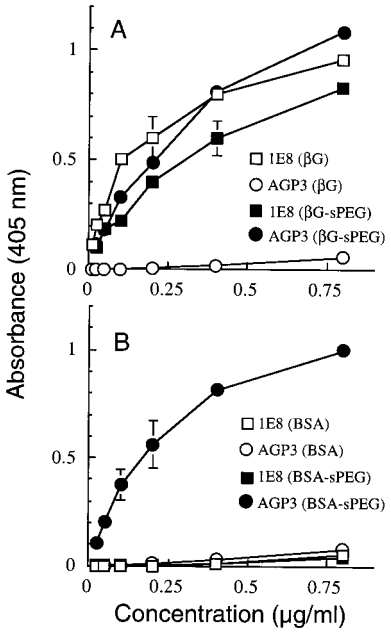

Figure 1. Binding specificity of 1E8 and AGP3. (A) 1E8 or AGP3 antibodies were incubated in microtiter plates coated with βG or βG−sPEG. (B) 1E8 or AGP3 were incubated in microtiter plates coated with BSA or BSA−sPEG. Binding of antibodies was detected by measuring the absorbance of wells at 405 nm as described in the Experimental Procedures. The mean absorbance values of triplicate determinations are shown. Bars, SE.

pH Dependence of βG Activity. Fifteen microliters of βG (4 μg/mL) was mixed in microtiter plates with 200 μl of βG buffer (Wang et al., 1992) with pH values ranging 3−10. Twenty-five microliters of *p*-nitrophenol β-D-glucuronide (32 mM) was added to the wells at 37 °C for 15 min, excess 1 N NaOH was added to adjust the pH to greater than 10, and absorbance was read at 405 nm in a microplate reader. To test the pH stability of βG, enzyme was incubated in βG buffer adjusted to pH values ranging 3−11 for 4 h at 37 °C before neutralization to pH 7.0 and assay of enzymatic activity.

Data 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

Antibodies against βG−PEG. Monoclonal antibodies against βG−sPEG were generated from spleen cells of mice that had received multiple injections of RH1−βG−PEG. mAb 1E8 (IgG1) and AGP3 (IgM) were selected for further characterization based on preliminary screening by ELISA. 1E8 bound to βG and, to a lesser extent, βG−sPEG (Figure 1A). The apparent affinity constants of 1E8 for βG and βG−sPEG were 0.83 and 1.8 nM, respectively (data not shown). In contrast, AGP3 bound βG−sPEG with an apparent affinity constant of 0.79 nM but did not bind βG (Figure 1A), suggesting that this antibody recognizes PEG chains present on βG−sPEG. AGP3 specificity for PEG was supported by the finding that it

Table 1. Characteristics of Galactose-Modified Monoclonal Antibodies

galactose added[a] (mg/mg)	conjugate	incorporated galactose groups/mAb		activity[d] (% control)	conjugate	incorporated galactose groups/mAb		activity[d] (% control)
		Fluor[b]	TNBS[c]			Fluor[b]	TNBS[c]	
0	1E8	0	0	100	AGP3	0	0	100
0.9	1E8−9G	12 ± 0	7 ± 0.6	100	AGP3−64G	60 ± 18	67 ± 10	72
1.8	1E8−19G	17 ± 2	21 ± 2	82	AGP3−130G	130 ± 4	130 ± 13	64
5.5	1E8−36G	35 ± 0.7	37 ± 2	72	AGP3−208G	210 ± 8	205 ± 21	48
10.9	1E8−46G	48 ± 0.1	44 ± 3	62	AGP3−285G	290 ± 0.1	280 ± 8	43
16.4	1E8−47G	47 ± 0.3	46 ± 2	66	AGP3−295G	310 ± 0.1	280 ± 7	42

[a] The amount of activated galactose added per milligram of antibody. [b] Determined by the fluorescamine method as described by Stocks et al. (1986). [c] Determine by the trinitrobenzesulfonic acid method as described in the Experimental Procedures. [d] Antigen-binding activity determined by ELISA.

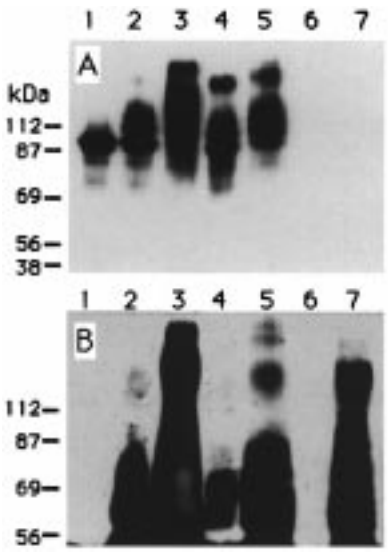

Figure 2. Immunoblot analysis of 1E8 and AGP3 binding. Proteins were electrophoresed in a 3−12.5% gradient reduced SDS−PAGE, transferred to nitrocellulose paper, and probed with 1E8 (A) or AGP3 (B) as described in the Experimental Procedures. Lane 1, βG; lane 2, βG−sPEG (2 mg of sPEG/mg of βG); lane 3, βG−sPEG (20 mg of sPEG/mg of βG); lane 4, βG-tPEG (2 mg of tPEG/mg of βG); lane 5, βG-tPEG (20 mg of tPEG/mg of βG); lane 6, BSA; lane 7, BSA−sPEG (20 mg of sPEG/mg of BSA). kDa, molecular mass in thousands.

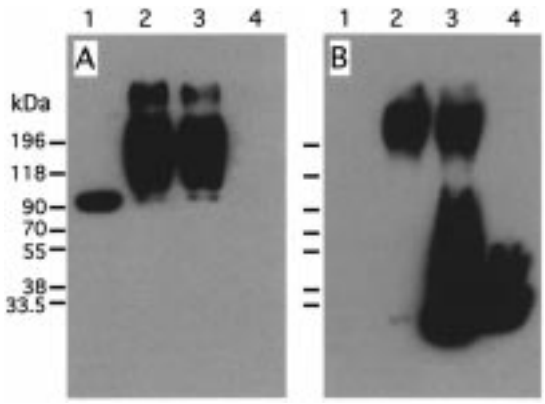

Figure 3. Immunoblot of different βG−PEG preparations. Samples were electrophoresed on a 8% reduced SDS−PAGE, transferred to nitrocellulose paper, and probed with 1E8 (A) or AGP3 (B) as described in the Experimental Procedures. Lane 1, βG; lane 2, βG−sPEG purified by gel filtration; lane 3, βG−sPEG purified by dialysis; lane 4, sPEG neutralized with excess glycine. kDa, molecular mass in thousands.

also bound BSA−sPEG but not BSA (Figure 1B). As expected, 1E8 bound neither BSA nor BSA−sPEG (Figure 1B). Neither mAb affected the activity of βG (results not shown).

The specificity of mAb binding was further examined by immunoblotting. Figure 2A shows that 1E8 detected βG and PEG-modified βG (lanes 1−5), but not BSA or BSA−sPEG (lanes 6 and 7). Figure 2B shows that AGP3, in contrast, did not bind βG (lane 1) or BSA (lane 6) but detected all proteins that incorporated PEG (βG−sPEG, βG−tPEG, and BSA−sPEG). The ability of AGP3 to bind either sPEG or tPEG-modified proteins demonstrates that this antibody binds to poly(ethylene glycol) rather than the linker between PEG and proteins.

Substantial amounts of PEG were present in βG−sPEG preparations purified by dialysis (Figure 3B, lane 3). AGP3 immunoblotting of sPEG after neutralization with glycine (Figure 3B, lane 4) produced a similar smeared band, indicating that the low molecular weight material in βG−sPEG corresponded to free PEG rather than degraded βG−sPEG fragments. Purification of βG−PEG by gel filtration on Sepharose S-200 HR resulted in the removal of free PEG (Figure 3B, lane 2). This βG−sPEG conjugate (Figure 3B, lane 2), containing 9.2 ± 0.3 sPEG groups/βG subunit and possessing 69% of original enzymatic activity, was employed in all subsequent experiments.

Galactose-Modification of Antibodies. 1E8 and AGP3 were modified with galactose to target their uptake by the asialoglycoprotein receptor on hepatocytes. Modification of 1E8 with increasing amounts of activated galactose resulted in the incorporation of up to 47 galactose residues/antibody (Table 1). Introduction of up to 20 galactose residues did not affect the binding of 1E8 to βG whereas greater incorporation of galactose decreased 1E8 activity by about 35%. A maximum of 290 galactose residues could be introduced into AGP3 (Table 1). The greater number of galactose residues incorporated into AGP3 primarily reflects the larger size of the IgM molecule; galactose incorporation for both 1E8 and AGP3 corresponded to 0.3 galactose moieties/kDa of antibody. AGP3 was more sensitive to galactose modification with a 2.4-fold decrease in antigen-binding activity at the highest levels of galactose incorporation.

The half-lives of galactose-modified antibodies were determined in BALB/c mice. Figure 4A shows that 1E8 was eliminated from serum with a half-life of 22 h. Introduction of increasing numbers of galactose residues into 1E8 resulted in progressively faster initial clearance rates. Even at the highest galactose incorporation, however, residual concentrations of 1−4 μg/mL 1E8−46G were present in the serum after 5 h. Unmodified AGP3 cleared from serum with a half-life of 13 h but in contrast to galactose-modified 1E8, almost no residual antibody could be detected in the circulation within 5 h after administration of AGP3 modified with greater than 130 galactose moieties (Figure 4B). On the basis of the elimination kinetics of the antibodies, 1E8−36G and AGP3−208G were selected for further study.

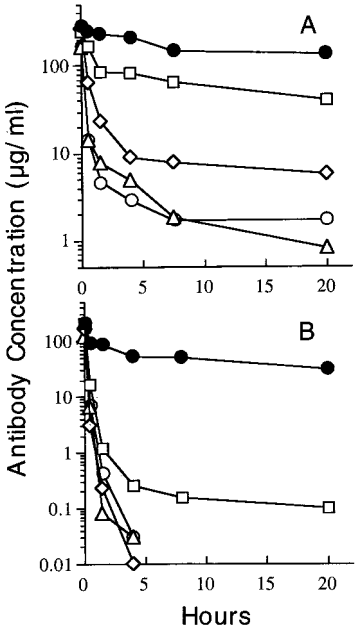

Figure 4. Elimination of galactose-modified antibodies from serum. Single BALB/c mice were i.v. injected with 200 μg of 1E8 (●), 1E8−9G (□), 1E8−19G (◇), 1E8−36G (△), or 1E8−46 (○) (A) or 200 μg of AGP3 (●), AGP3−64G (□), AGP3−130G (◇), AGP3−208G (△), or AGP3−285G (○) (B). The concentrations of antibodies in serum samples taken at the indicated times were measured as described in the Experimental Procedures.

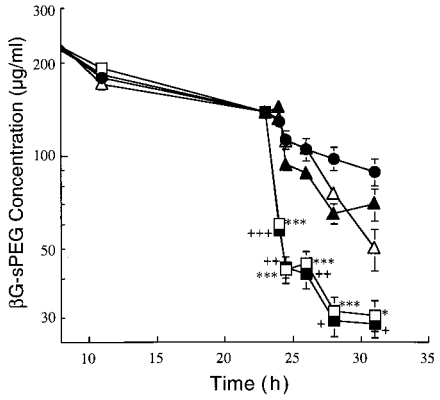

Figure 5. Clearance of βG−PEG by antibodies. Mice injected with 200 μg of βG−sPEG at time 0 were i.v. injected 23 h later with PBS (●) or 250 μg of 1E8 (△), 1E8−36G (▲), AGP3 (□), or AGP3−208G (■). The concentration of βG−sPEG in serum samples was measured and normalized as described in the Experimental Procedures. Mean concentrations from three mice are shown. Significant differences between AGP3 and 1E8 clearance are indicated: (*) $p \leq 0.05$; (**) $p \leq 0.005$; (***) $p \leq 0.0005$. Significant differences between AGP3−208G and 1E8−36G clearance are indicated: (+) $p \leq 0.05$; (++) p ≤ 0.005; (+++) $p \leq 0.0005$. Bars, SE.

In Vivo Clearance of βG−PEG. The ability of antibodies to clear βG−sPEG from the circulation was examined by i.v. injecting mice with 200 μg of βG−sPEG followed 24 h later with 250 μg of unmodified or galactose-modified antibody. Figure 5 shows that both 1E8 and AGP3 accelerated the clearance of βG−sPEG from the circulation. 1E8−36G cleared significantly more βG−sPEG than 1E8 at early times but by 8 h after antibody administration there was no significant difference between these antibodies. AGP3 and AGP3−208G cleared significantly more βG−sPEG than either 1E8 or 1E8−36G at all times examined. βG−sPEG was cleared equally well by AGP3 and AGP3−208G under these experimental conditions.

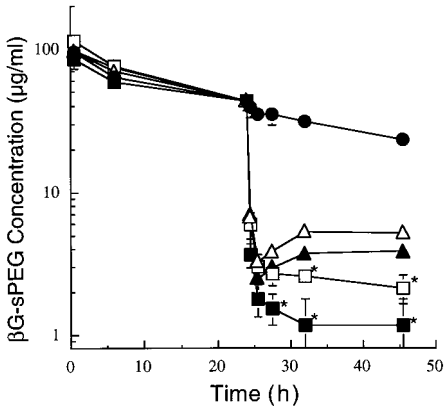

Figure 6. Dose dependence of AGP3 clearance of βG−PEG. Groups of three mice were injected at time 0 with 50 μg of βG−sPEG followed 24 h later by PBS (●), 150 μg of AGP3 (□), 150 μg of AGP3−208G (△), 300 μg of AGP3 (■), or 300 μg of AGP3−208G (▲). The concentration of βG−PEG in serum samples was measured and normalized as described in the Experimental Procedures. Mean concentrations from three mice are shown. Significant differences between AGP3 and AGP3−208G groups are indicated; (*) $p \leq 0.05$. Bars, SE.

Table 2. Clearance of 5A8-βG−PEG by AGP3[a]

		AGP3 clearance	
time (h)	no clearance (μg/mL)	(μg/mL)	reduction[b](fold)
24	24 ± 2.1	37 ± 1.5	1.0
26	18 ± 3.5	0.10 ± 0.001	280
48	6.1 ± 0.7	0.01 ± 0	940

[a] Groups of 3 mice were i.v. injected with 175 μg of 5A8-βG−PEG. At 24 and 25 h, mice were injected with PBS (no clearance) or 300 μg of AGP3. The concentration of 5A8-βG−PEG in serum was determined by measuring βG activity. [b] The ratio of 5A8-βG−PEG concentration in serum without clearance to the concentration with clearance, normalized for the difference in conjugate concentrations at 24 h.

We examined whether increasing the ratio of AGP3 to βG−sPEG would improve clearance. Figure 6 shows that i.v. injection of 150 or 300 μg of AGP3 or AGP3−208G 24 h after i.v. injection of 50 μg of βG−sPEG resulted in rapid removal of 90−95% of the enzyme within 1.5 h. The concentration of βG−sPEG in the circulation, however, rebounded from a minimum of about 2 μg/mL to 4−5 μg/mL 8 h after injection of AGP3−208G. In contrast, the concentration of βG−sPEG decreased to 1.0 μg/mL 8 h after injection of 300 μg of AGP3. Although a dose of 300 μg of AGP3 appeared to more completely clear βG−sPEG compared to 150 μg of AGP3, the difference was only significant ($p \leq 0.05$) 1.5 h after antibody administration. In contrast, clearance of βG−sPEG with both 150 and 300 μg of AGP3 was significantly ($p \leq 0.05$) better than clearance with either dose of AGP3−208G at all times. AGP3 also efficiently cleared 5A8−βG−PEG, reducing the serum concentration of conjugate by 280-fold within 2 h (Table 2).

Prodrug Toxicity After Clearance of βG−sPEG. Clearance of circulating βG−sPEG should reduce systemic activation of the glucuronide prodrug BHAMG, thereby reducing toxicity. Accumulation of cleared immune complexes in organs, however, could result in tissue damage. Organ tissue sections from mice that were injected with PBS or βG−sPEG followed 24 h later by AGP3 and then BHAMG were therefor examined after 3 or 10 days. Figure 7 shows that injection of AGP3 and BHAMG as well as clearance of βG−sPEG with AGP3 before BHAMG administration did not cause obvious

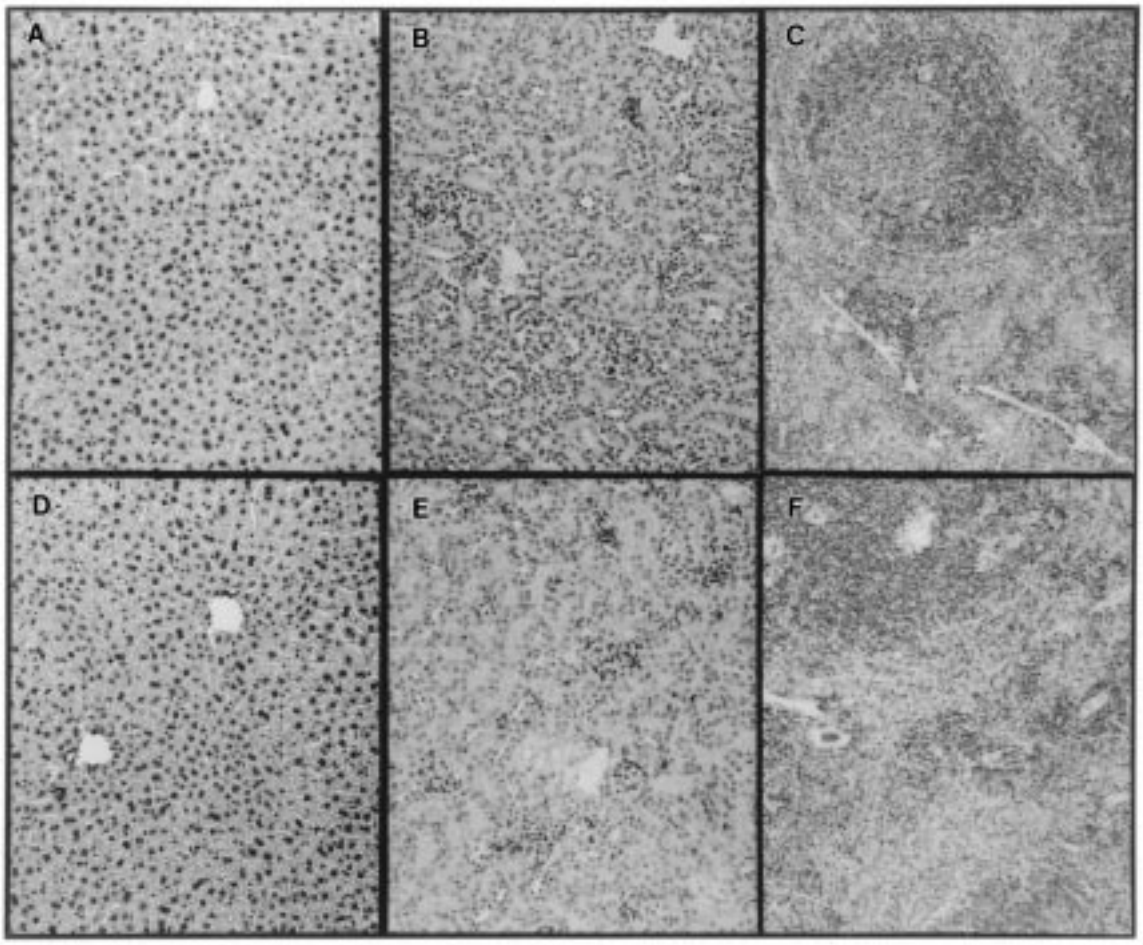

Figure 7. BHAMG-induced organ pathology after βG−PEG clearance. Mice were injected with PBS (A−C) or βG−sPEG (D−F) on day 1. Mice were i.v. injected with AGP3 and BHAMG 24 and 30 h later, respectively. Tissue sections of liver (A, D), kidney (B, E), and spleen (C, F) recovered 10 days later were stained with hematoxylin and eosin.

damage to the liver, spleen, or kidney 10 days after BHAMG administration. Similarly, no tissue damage was observed 3 days after prodrug administration (results not shown).

The toxicity associated with clearance and prodrug administration was also assessed by measuring the weight and blood cells of mice. Administration of AGP3 and 15 mg/kg BHAMG (control group) produced only a small transient decrease in the weight of mice (Figure 8A). In contrast, injection of 50 μg of βG−sPEG 24 h before administration of BHAMG caused significant weight loss and resulted in the death of 1 of 4 mice. Clearance of βG−sPEG with 300 μg of AGP3 or 2 injection of 150 μg of AGP3−208G before prodrug administration prevented significant weight loss compared to the control group. Mice that were cleared with one 300 μg dose of AGP3−208G before injection of BHAMG lost significantly more weight than control mice after day 8, but the loss was much less than in the uncleared mice. Figure 8B shows that the numbers of RBC, PLT, and RBC were significantly reduced in mice injected with βG−sPEG and BHAMG compared to mice injected with AGP3 and BHAMG (control mice). In contrast, the numbers of RBC, WBC, and PLT in mice in which βG−sPEG was cleared with 300 μg of AGP3 before BHAMG administration were not significantly different from control mice. Clearance of βG−sPEG with AGP3−208G before injection of prodrug reduced but did not eliminate hematological toxicity.

AGP3/βG−PEG immune complexes may be routed to the acidic environment of lysosomes after clearance. The effect of pH on βG activity was examined by incubating βG at various pH values for 4 h before neutralization and assay of activity at pH 7.0. βG was irreversibly deactivated at pH values less than 5.0 or greater than 9.0 (Figure 9).

DISCUSSION

We have previously shown that PEG modification of βG increases the tumor localization of antibody−βG conjugates for targeted activation of glucuronide prodrugs (Cheng et al., 1997). PEG-modified βG conjugates, however, are slowly eliminated from the circulation, resulting in low tumor-to-blood ratios. We therefore generated mAbs against βG and PEG and investigated whether these antibodies could accelerate the clearance of βG−sPEG from blood.

mAb 1E8 and AGP3 were modified with galactose to direct uptake of immune complexes by asialoglycoprotein receptors on liver cells (Thornburg et al., 1980; Mattes, 1987). Binding of circulating βG−sPEG by 1E8−36G or AGP3−208G was expected to result in rapid endocytosis, routing to lysosomes and catabolism of enzyme (Ciechanover et al., 1983; Geuze et al., 1983), thereby preventing systemic activation of glucuronide prodrugs. In agreement with previous studies (Mattes, 1987; Sharma et al., 1990), extensive galactose modification of 1E8 and AGP3 (up to 47 and 290 residues, respectively) did not dramati-

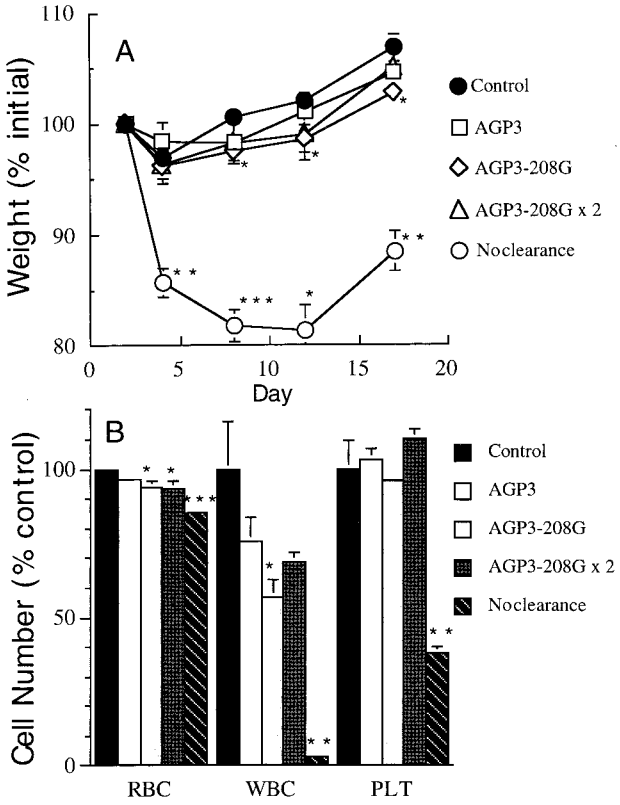

Figure 8. Weight and hematological toxicity of BHAMG after clearance of βG−PEG. Groups of 4 mice were i.v. injected at time zero with PBS (control) or βG−sPEG. Twenty-four hours later, mice received i.v. injections of 300 μg of AGP3 (control and AGP3 groups), 300 μg of AGP3−208G, 150 μg of AGP3−208G (×2, 4 h delay), or PBS (no clearance). Six hours later, all mice were i.v. injected with BHAMG. (A) The time course of mouse weight (% of initial weight) and (B) mean numbers of WBC, RBC, and platelets on day 8 are shown. Significant differences between the control and experimental groups are indicated: (*) $p \leq 0.05$; (**) $p \leq 0.005$; (***) $p \leq 0.0005$. Bars, SE.

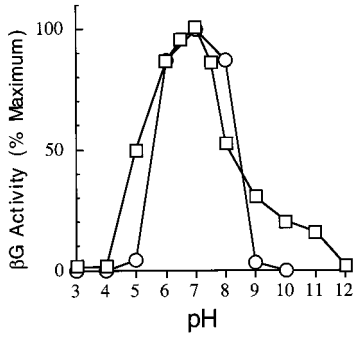

Figure 9. pH sensitivity of βG. βG was assayed for enzymatic activity at various pH values (□) or incubated at the indicated pH for 4 h before neutralization and assay at pH 7.0 (○). Results represent percentage of maximum activity.

cally alter the antigen-binding activity of the antibodies. Increasing the level of galactose residues per antibody did not greatly affect the initial rate of clearance, but did increase the total amount of antibody that was removed from the circulation. For example, the concentrations of 1E8−9G, 1E8−19G, and 1E8−36G in serum were 45, 5.3, and 1.2% of 1E8 levels after 8 h (Figure 4). The galactose-modified antibodies that were not removed from the circulation within the first 5 h exhibited similar elimination kinetics as unmodified 1E8, indicating that a critical number of galactose residues was required for efficient uptake by the asialoglycoprotein receptor, similar to the results found for galactosylated BSA and superoxide dismutase (Nishikawa et al., 1995). The more efficient uptake of galactosylated AGP3 (=99.5% by 4 h for ϵ 130 galactose moieties) compared to galactose-modified 1E8 was unlikely due to differences in the surface density of galactose groups since galactose incorporation was similar based on the molecular weights of 1E8 and AGP3. The asialoglycoprotein receptor is a hetero-oligomer composed of two types of polypeptide chains (Lodish, 1991). High-affinity binding of ligands involves the interaction of three galactose residues in ligands with sites in both polypeptide chains (Rice et al., 1990). Specific orientation of the galactose residues is required for high-affinity receptor binding (Lee et al., 1983; Townsend et al., 1986), suggesting that the orientation of incorporated galactose groups in the pentameric structure of IgM may promote high-affinity binding to the asialoglycoprotein receptor.

AGP3 cleared βG−sPEG more rapidly and completely than 1E8. Hindered binding of 1E8 by PEG chains on βG−sPEG may have contributed to the high residual serum concentrations of βG−sPEG after 1E8 administration. The pentameric structure of AGP3 combined with the presence of multiple PEG chains on each βG tetramer may have promoted the formation of large immune complexes, which are cleared more rapidly than small immune complexes (Roitt et al., 1989). AGP3 appears to produce more rapid and complete clearance than other antibodies previously employed for clearance, which have been limited to IgG fractions of polyclonal antiserum (Bradwell et al., 1983; Sharkey et al., 1984; Pedley et al., 1989; Begent et al., 1989) or monoclonal IgG antibodies (Sharma et al., 1990; Kerr et al., 1993; Haisma et al., 1995; Wallace et al., 1994). Polyclonal IgG antibodies reduced radioimmunoconjugate levels in serum from around 10-fold (Bradwell et al., 1983; Sharkey et al., 1984; Begent et al., 1989) to 130-fold (Pedley et al., 1989) in 24 h whereas monoclonal IgG antibodies reduced the serum levels of antibody−enzyme conjugates from 3−10-fold in a few hours (Sharma et al., 1990; Haisma et al., 1995) to 40−60-fold in 24 h (Kerr et al., 1993; Wallace et al., 1994). In comparison, AGP3 reduced the serum concentration of 5A8−βG−PEG by 280-fold in 2 h and 940-fold in 24 h. Although it is difficult to directly compare clearance results employing different immunoconjugates and experimental conditions, these results suggest that IgM may clear immunoconjugates from blood more rapidly and completely than IgG.

Comparison of AGP3 and AGP3−208G clearance demonstrated that both antibodies initially cleared βG−sPEG with similar rapid kinetics, but that AGP3 removed significantly more βG−sPEG from the circulation than AGP3−208G. The better clearance of unmodified AGP3 can be attributed to the prolonged half-life of AGP3 ($\tau_{1/2}$=13 h) compared to AGP3−208G ($\tau_{1/2}$ < 1 h). βG−sPEG released from normal tissues after rapid removal from blood could be bound and cleared by free AGP3 remaining in the blood pool whereas the rapid endocytosis of both free and complexed AGP3−208G prevented further formation of complexes with βG−sPEG. This behavior is clearly evident in Figure 6 where the concentration of βG−sPEG in the circulation rebounded after an initial period of rapid clearance by AGP3−208G.

One concern of employing IgM to clear proteins is that the immune complexes could be deposited in organs and cause tissue damage. In addition, deposition of βG−PEG on the surface of cells could result in prodrug activation and tissue toxicity. Tissue damage, however, was not

observed after clearance of βG−sPEG with AGP3 and subsequent administration of BHAMG. In addition, clearance of βG−sPEG with AGP3 prevented weight loss and hematological toxicity associated with systemic activation of BHAMG by βG−PEG in the circulation. Soluble IgM immune complexes are primarily removed from the circulation and catabolized by the mononuclear phagocyte system in the liver, spleen, and lungs (Roitt et al., 1989), possibly by receptor-mediated binding of high mannose oligosaccharides exposed upon conformational changes in IgM after binding to antigen (Day et al., 1980). The low toxicity of BHAMG after clearance of βG−sPEG with AGP3 indicates that immune complexes formed between AGP3 and βG−sPEG are rapidly internalized where βG is inactivated, degraded or inaccessible to prodrug. Routing of βG−sPEG to lysosomes is expected to result in loss of enzymatic activity because βG is irreversibly inactivated at pH values lower than 5.0. Deactivation of βG in lysosomes may prevent accumulation of active enzyme and prodrug activation in the liver as observed after antibody-mediated clearance of carboxypeptidase G2 conjugates (Rogers et al., 1995).

PEG modification is a useful technique to improve the stability, prolong serum half-life and decrease the antigenicity and immunogenicity of proteins (Delgado et al., 1992). Because AGP3 binding to PEG is protein independent, this antibody should be generally applicable to the clearance of PEG-modified immunoconjugates. Clearance of immunoconjugates from the circulation can also result in decreased tumor accumulation (Pedley et al., 1989; Sharkey et al., 1992; Kerr et al., 1993), likely due to the generation of a concentration gradient of conjugate from tumor to blood, allowing immunoconjugates that dissociate from tumor antigen to be released into the blood. The large size of IgM may retard passage into the tumor interstitial space, minimizing interactions between localized immunoconjugate and clearing antibody. In addition, the rapid clearance achieved with AGP3 may allow administration of prodrug or other targeting moieties before significant immunoconjugate is released from the tumor. The application of AGP3 for clearing B72.3−βG−PEG conjugates for glucuronide prodrug therapy of colorectal cancer is currently under investigation.

ACKNOWLEDGMENT

Supported by a Grant (NSC88-2314-B001-036) from the National Science Council, Republic of China.

LITERATURE CITED

(1) Beatty, J. D., Beatty, B. G., and Vlahos, W. G. (1987) Measurement of monoclonal antibody affinity by noncompetitive enzyme immunoassay. *J. Immunol. Methods 100*, 173−179.

(2) Begent, R. H. J., Ledermann, J. A., Green, A. J., Bagshawe, K. D., Riggs, S. J., Searle, F., Keep, P. A., Adam, T., Dale, R. G., and Glaser, M. G. (1989) Antibody distribution and dosimetry in patients receiving radiolabeled antibody therapy for colorectal cancer. *Br. J. Cancer 60*, 406−412.

(3) Bradwell, A. R., Vaughan, A., Fairweather, D. S., and Dykes, P. W. (1983) Improved radioimmunodetection of tumours using second antibody. *Lancet i*, 247.

(4) 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.

(5) 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 β-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs. *Cancer Immunol. Immunother. 44*, 305−315.

(6) Ciechanover, A., Schwartz, A. L., and Lodish, H. F. (1983) The asialoglycoprotein receptor internalizes and recycles independently of the transferrin and insulin receptors. *Cell 32*, 267−275.

(7) 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.

(8) Delgado, C., Francis, G. E., and Fisher, D. (1992) The uses and properties of PEG-linked proteins. *Crit. Rev. Therapeut. Drug Carr. Systems 9*, 249−304.

(9) Fujimori, K., Covell, D. G., Fletcher, J. E., and Weinstein, J. N. (1990) A modeling analysis of monoclonal antibody percolation through tumors: a binding-site barrier. *J. Nucl. Med. 31*, 1191−1198.

(10) Geuze, H. J., Slot, J. W., Strous, G. J., Lodish, H. F., and Schwartz, A. L. (1983) Intracellular site of asialoglycoprotein receptor−ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell 32*, 277−287.

(11) Goldenberg, D. M., Sharkey, R. M., and Ford, E. (1987) Anti-antibody enhancement of iodine-131 anti-CEA radioimmunodetection in experimental and clinical studies. *J. Nucl. Med. 28*, 1604−1610.

(12) Guilarte, T. R. (1985) Radiometric-microbiological assay of biotin in human plasma. *Ann. N. Y. Acad. Sci. 447*, 398−399.

(13) Habeeb, A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem. 14*, 328−336.

(14) 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.

(15) Ishikawa, E., Hashida, S., Kohno, T., and Ohtaki, S. (1987) Modification of monoclonal antibodies with enzymes, biotin, and fluorochromes and their applications. *Monoclonal Antibody Production Techniques and Applications* (L. B. Schook, Ed.) pp 113−139, Marcel Dekker, Inc., New York.

(16) Kerr, D. E., Garrigues, U. S., Wallace, P. M., Hellström, K. E., Hellström, 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.

(17) Klibanov, A. L., Martynov, A. V., Slinkin, M. A., Sakharov, I. Y., Smirnov, M. D., Muzykantov, V. R., Danilov, S. M., and Torchilin, V. P. (1988) Blood clearance of radiolabeled antibody: enhancement by lactosamination and treatment with biotin−avidin or anti-mouse antibodies. *J. Nucl. Med. 29*, 1951−1956.

(18) Kobayashi, H., Sakahara, H., Endo, K., Hosono, M., Yao, Z. S., Toyama, S., and Konishi, J. (1995) Comparison of the chase effects of avidin, streptavidin, neutravidin, and avidin-ferritin on a radiolabeled biotinylated antitumor monoclonal antibody. *Jpn. J. Cancer Res. 86*, 310−314.

(19) Lee, Y. C., Townsend, M. R., Hardy, J., Lönngren, J., Arnarp, M., Haraldsson, M., and Lönn, H. (1983) Binding of synthetic oligosaccharides to the hepatic Gal/GalNAc lectin. Dependence on fine structural features. *J. Biol. Chem. 258*, 199−202.

(20) Lodish, H. F. (1991) Recognition of complex oligosaccharides by the multi-subunit asialoglycoprotein receptor. *Trends Biol. Sci. 16*, 374−377.

(21) Maloney, D. G., Kaminski, M. S., Burowski, D., Haimovich, J., and Levy, R. (1985) Monoclonal anti-idiotype antibodies against the murine B cell lymphoma 38C13: Characterization and use as probes for the biology of the tumor in vivo and in vitro. *Hybridoma 4*, 191−209.

(22) Marshall, D., Pedley, R. B., Melton, R. G., Boden, J. A., and Begent, R. H. J. (1995) Galactosylated streptavidin for improved clearance of biotinylated intact and $F(ab')_2$ fragments of an anti-tumour antibody. *Br. J. Cancer 71*, 18−24.

(23) Martin, J., Stribbling, S. M., Poon, G. K., Begent, R. H., Napier, M., Sharma, S. K., and Springer, C. J. (1997) Antibody-directed enzyme prodrug therapy: pharmacokinetic and plasma levels of prodrug and drug in a phase I clinical trail. *Cancer Chemother. Pharmacol. 40*, 189−201.

(24) Mattes, M. J. (1987) Biodistribution of antibodies after intraperitoneal or intravenous injection and effect of carbohydrate modifications. *J. Natl. Cancer Inst. 79*, 855−863.

(25) Nishikawa, M., Miyazaki, C., Yamashita, F., Takakura, Y., and Hashida, M. (1995) Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. A*m. J. Physiol. 268*, G849−G856.

(26) Ong, G. L., Ettenson, D., Sharkey, R. M., Marks, A., Baumal, R., Goldenberg, D. M., and Mattes, M. J. (1991) Galactose-conjugated antibodies in cancer therapy: properties and principles of action. *Cancer Res. 51*, 1619−26.

(27) Pedley, R. B., Dale, R., Boden, J. A., Begent, R. H. J., Keep, P. A., and Green, A. J. (1989) The effect of second antibody clearance on the distribution and dosimetry of radiolabeled anti-CEA antibody in a human colonic tumor xenograft model. *Int. J. Cancer 43*, 713−718.

(28) Rice, K. G., Weisz, O. A., Barthel, T., Lee, R. T., and Lee, Y. C. (1990) Defined geometry of binding between triantennary glycopeptides and the asialoglycoprotein receptor of rat hepatocytes. *J. Biol. Chem 265*, 18429−18434.

(29) 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.

(30) Roffler, S. R., Chan, J., and Yeh, M. Y. (1994) Potentiation of radioimmunotherapy by inhibition of topoisomerase I. *Cancer Res. 54*, 1276−1285.

(31) 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.

(32) Roit, I., Brostoff, J., and Male, D. (1989) Immunology, pp 21.5−21.9, Gower Medical Publishing, London.

(33) Sharkey, R. M., Primus, F. J., and Goldenberg, D. M. (1984) Second antibody clearance of radiolabeled antibody in cancer radioimmunodetection. *Proc. Natl. Acad. Sci. U.S.A. 81*, 2843−2846.

(34) 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 synegeic anti-idiotype antibody in tumor-bearing nude mice. *Int. J. Cancer 51*, 266−273.

(35) 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 localization in a xenograft model. *Br. J. Cancer 61*, 659−662.

(36) Sharma, S. K., Bagshawe, K. D., Burke, P. J., Boden, J. A., Rogers, G. T., Springer, C. J., Melton, R. G., and Sherwood, R. F. (1994) Galactosylated antibodies and antibody-enzyme conjugates in antibody-directed enzyme prodrug therapy. *Cancer 73*, 1114−20.

(37) Stella, M., DeNardo, P., Paganelli, G., Magnani, P., Mangili, F., Sassi, I., Baratti, D., Gini, P., Zito, F., and Cristallo, M. (1994) Avidin−biotin system in radioimmunoguided surgery for colorectal cancer. Advantages and limits. *Dis. Colon Rectum 37*, 335−343.

(38) Stocks, S. J., Jones, A. J. M., Ramey, C. W., and Brooks, D. E. (1986) A fluorometric assay of the degree of modification of protein primary amines with poly(ethylene glycol). *Anal. Biochem. 154*, 232−234.

(39) Thornburg, R. W., Day, J. F., Baynes, J. W., and Thorpe, S. R. (1980) Carbohydrate-mediated clearance of immune complexes from the circulation. A role for galactose residues in the hepatic uptake of IgG-antigen complexes. *J. Biol. Chem. 255*, 6820−6825.

(40) Thomas, G. D., Chappell, M. J., Dykes, P. W., Ramsden, D. B., Godfrey, K. R., Ellis, J. R. M., and Bradwell, A. R. (1989) Effect of dose, molecular size. affinity, and protein binding on tumor uptake of antibody or ligand: A mathematical model. *Cancer Res. 49*, 3290−3296.

(41) Townsend, R. R., Hardy, M. R., Wong, T. C., and Lee, Y. C. (1986) Binding of N-linked bovine fetuin glycopeptides to isolated rabbit hepatocytes: Gal/GalNAc hepatic lectin discrimination between Galβ(1,4)GalNAc and Galβ(1,3)GalNAc in a triantennary structure. *Biochemistry 25*, 5716−5725.

(42) 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.

(43) 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.

(44) Wolf, B., Hymes, J., and Heard, G. S. (1990) Biotinidase. *Methods Enzymol. 184*, 103−111.

(45) Yao, Z., Zhang, M., Kobayashi, H., Sakahara, H., Nakada, H., Yamashina, I., and Konishi, J. (1995) Improved targeting of radiolabeled streptavidin in tumors pretargeted with biotinylated monoclonal antibodies through an avidin chase. *J. Nucl. Med. 36*, 837−841.

(46) Yeh, M. Y, Hellström, I., Brown, J. P., Warner, G. A., Hansen, J. A., and Hellström, K. E. (1979) Cell surface antigens of human melanoma identified by monoclonal antibody. *Proc. Natl. Acad. Sci. U.S.A. 76*, 2927−1931.

(47) Yeh, M. Y., Roffler, S. R., and Yu, M. H. (1992) Doxorubicin-monoclonal antibody conjugate for therapy of human cervical carcinoma. *Int. J. Cancer 51*, 274−282.

(48) Zhang, M., Sakahara, H., Yao, Z., Saga, T., Nakamoto, Y., Sato, N., Nakada, H., Yamashina, I., and Konishi, J. (1997) Intravenous avidin chase improved localization of radiolabeled streptavidin in intraperitoneal xenograft pretargeted with biotinylated antibody. *Nucl. Med. Biol. 24*, 61−64.

BC980143Z