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CURE OF MALIGNANT ASCITES AND GENERATION OF PROTECTIVE IMMUNITY BY MONOCLONAL ANTIBODY–TARGETED ACTIVATION OF A GLUCURONIDE PRODRUG IN RATS

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We examined the *in vivo* efficacy of targeting β-glucuronidase (βG) to activate a glucuronide prodrug (BHAMG) of p-hydroxyaniline mustard (pHAM) at hepatoma ascites in Sprague-Dawley rats. Injection i.p. of 500 μg RH1-βG, a conjugate formed between recombinant βG and monoclonal antibody RH1 with specificity for an antigen expressed on AS-30D rat hepatoma cells, into rats bearing AS-30D ascites resulted in the accumulation of 54 μg conjugate per 10^9 tumor cells after 2 hr. Ascites fluid and serum contained 0.53 and 0 μg/ml, respectively, RH1-βG 2 hr after injection of the conjugate. Conjugate binding to AS-30D cells was heterogeneous and non-saturated, as determined by flow cytometry. BHAMG was less toxic than pHAM to SD rats based on measures of animal mortality, weight loss and hematological toxicity. Treatment of rats bearing established hepatoma ascites with 500 μg RH1-βG followed 2 hr later with a single i.p. injection of 30 mg/kg BHAMG or 3 i.p. injections of 10 mg/kg BHAMG 2, 3 and 4 hr later resulted in the cure of 6/8 and 8/8 animals, respectively. Treatment with BHAMG or pHAM alone did not produce cures, whereas treatment with a control antibody–βG conjugate and BHAMG produced significantly greater hematological toxicity compared to treatment with RH1-βG and BHAMG. All cured rats were completely protected from rechallenge with 2×10^7 AS-30D cells, indicating that successful treatment of animals induced protective immunity. *Int. J. Cancer* 73:392–402, 1997.

Treatment of malignant ascites represents a difficult problem most often encountered in advanced or recurrent cases of ovarian, endometrial, breast, colon, gastric and pancreatic carcinomas. Although a variety of modalities have been employed for the treatment of malignant ascites, the common use of peritoneovenous shunts (Gough and Balderson, 1993) for the palliation of ascites indicates that more effective methods are required.

Increasing the specificity of chemotherapy may help to improve the efficacy of cancer treatment. One approach to increase cancer drug selectivity is to target an antibody–enzyme conjugate to tumor cells and then treat with a prodrug that can be converted into a cytotoxic agent by the enzyme (Bagshawe *et al.,* 1988; Senter *et al.,* 1988). Systemic distribution of the prodrug should result in little toxicity, whereas enzymatic generation of the active drug at cancer cells is expected to provide high localized concentrations of the cytotoxic agent at the tumor site.

Several enzyme–prodrug combinations currently are being investigated for cancer therapy (Melton and Sherwood, 1996). We have developed a glucuronide prodrug (BHAMG) that can be activated to p-hydroxyaniline mustard (pHAM) by β-glucuronidase (βG) linked to monoclonal antibodies (MAbs) (Roffler *et al.,* 1991; Wang *et al.,* 1992). βG-activated glucuronide prodrugs possess potential advantages for cancer therapy. The low concentration of βG in human serum (Stahl and Fishman, 1984) and the observation that glucuronide conjugates are major metabolites of many drugs in humans indicate that glucuronide prodrugs should be stable in the blood after i.v. administration. In addition, βG exhibits optimal activity at physiological pH (Jefferson *et al.,* 1986) and the low specificity of βG for aglycones conjugated with the glucuronide group (Stahl and Fishman, 1984) has allowed the development of a variety of glucuronide prodrugs (Bosslet *et al.,* 1994; Haisma *et al.,* 1994; Nolen *et al.,* 1995). Glucuronide prodrugs also possess increased water solubility (Haisma *et al.,* 1992), which may allow improved formulation of insoluble anti-neoplastic agents.

We report on the efficacy of preferentially activating BHAMG at tumor cells pre-targeted with antibody–βG conjugates in a rat hepatoma ascites model. One concern of employing glucuronide prodrugs for cancer therapy is that endogenous βG present in non-tumor tissues could produce high systemic concentrations of cytotoxic drugs, which could decrease treatment specificity. Several tissues also express uridine 5′-diphosphoglucuronyl transferases (UDPGT), a family of xenobiotic detoxification enzymes that can transfer a glucuronide group from UDP-glucuronic acid to aglycones such as pHAM and may counter-balance glucuronide activation by endogenous βG. The activities of βG and UDPGT, therefore, were measured in isolated rat tissues to determine which organs may be susceptible to non-specific prodrug activation. Tissue damage by endogenously activated prodrug in rats was determined by histopathological examination of tissue sections after i.p. injection of BHAMG. The hematological toxicity of pHAM and BHAMG also was examined due to the well-known toxicity of alkylating agents to blood cells. A conjugate formed between βG and MAb RH1, an IgG_{2a} MAb that binds to a 32 kDa antigen expressed on the surface of AS-30D hepatoma cells (Roffler *et al.,* 1994), retained antigen-binding and enzymatic activities and was able to localize at AS-30D hepatoma ascites cells. Treatment of rats bearing established hepatoma ascites by sequential i.p. injection of RH1-βG and BHAMG resulted in the cure of 14 of 16 animals. In addition, cured rats were protected from subsequent challenge with AS-30D cells.

MATERIAL AND METHODS

Reagents

BHAMG and pHAM were synthesized as described by Roffler *et al.* (1991). Succinimidyl succinate monomethoxy poly(ethylene glycol) (m.w. = 5,000), CHAPS, p-nitrophenyl β-D-glucuronide (PNPG), p-nitrophenol and βG (EC 3.2.1.31) from *Escherichia coli* (type X-A) were purchased from Sigma (St. Louis, MO). Sephadex G-25 and Sephacryl S-300 HR gels were from Pharmacia (Uppsala, Sweden). Succinimidyl-4-(n-maleimidomethyl)cyclohexane 1-carboxylate was from Pierce (Rockford, IL). Recombinant βG was produced as described by Cheng *et al.* (1997).

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Cell lines

N1S1 rat hepatoma cells and NOR 10 mouse normal muscle cells were obtained from the ATCC (Rockville, MD). The AS-30D rat hepatoma cell line was generously provided by Dr. J.P. Chang (Institute of Zoology, Academia Sinica, Taipei, Taiwan). Cells were cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 5% heat-inactivated bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Antibodies

RH1 MAb, an IgG_{2a} murine antibody that binds to a surface antigen expressed on AS-30D hepatoma cells, was generated in our laboratory (Roffler *et al.*, 1994). Hybridoma H16-L10-4R5, which secretes an IgG_{2a} antibody (MAb HB65) against the nucleoprotein of influenza type A virus, was obtained from the ATCC. MAbs were purified from ascites produced in BALB/c mice by protein-A affinity chromatography. Polyclonal antibodies against βG were obtained by immunizing rabbits with recombinant βG. Secondary antibodies were from Organon (Durham, NC).

Animals

Sprague-Dawley rats, BALB/c mice and scid mice were obtained from and maintained in the animal room of the Institute of Biomedical Sciences, Academia Sinica. Animal experiments were performed in accordance with institute guidelines.

Lethal toxicity of drugs

Single i.p. injections of BHAMG (30, 50, 60, 100 or 150 mg/kg) dissolved in PBS or pHAM (0, 5, 7.5, 10 or 20 mg/kg) dissolved in DMSO/propylene glycol (PG, 1:5 vol/vol) were administered to groups of 3–5 rats. Animal survival was monitored for 45 days.

Organ toxicity of drugs

Cellular activities of enzymes involved in the metabolism of glucuronide prodrugs were measured in tissue homogenates of organs obtained from SD rats weighing 250–300 g. Rats were killed and blood was immediately centrifuged at 4°C to isolate serum. Organs were placed on ice, cut into pieces and homogenized in 20 mM bis-Tris buffer, pH 6.0, containing 0.1% Triton X-100 in an ice-bathed glass homogenizer. Tissue homogenates were stored at −76°C until analysis. Three rats were used to determine the average activities of βG and UDPGT as described (Wang *et al.*, 1992).

Groups of 3 specific pathogen-free SD rats were i.p.-injected with PBS or 120 mg/kg BHAMG in PBS on day 0. The spleen, liver, kidney, intestines and lungs were removed from 1 rat in each group on days 2, 5 and 9 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.

Hematological toxicity of drugs

Groups of 4 SD rats were i.p.-injected with 30 or 60 mg/kg BHAMG in PBS or 7.5 mg/kg pHAM in DMSO/PG (1:5 vol/vol) on day 0. Blood samples were collected from each rat 2 days before drug treatment (day −2) and on days 3 and 6 and once every week thereafter. Fifty microliters of blood from the tail vein were collected into Labcraft disposable 100 µL calibrated pipets that had been pre-filled with 50 µl PBS containing 1.5 mg/ml EDTA. Blood was expelled immediately into microfuge tubes containing 50 µl of the same buffer and the number of platelets, lymphocytes and red blood cells counted with a Coulter T-540 cell counter (Hialeah, FL). Liver function was monitored by assaying for serum γ-glutamyl transferase according to the manufacturer's instructions (Sigma).

Antibody–βG conjugates

The conjugates RH1-βG and HB65-βG were formed by linking recombinant βG to MAbs RH1 or HB65 *via* a thioether bond, as described (Wang *et al.*, 1992). Protein concentrations were measured by the bicinchoninic acid assay (Pierce). βG activity of conjugates was assayed as described (Wang *et al.*, 1992). Antigen binding and βG activities of conjugates were determined by ELISA with the substrate PNPG in 96-well microtiter plates coated with AS-30D or NOR 10 cells (Yeh *et al.*, 1992).

RH1–βG tumor localization

Groups of 4 Sprague-Dawley rats weighing 250–300 g were i.p.-injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 7, rats received a single i.p. injection of 500 µg RH1-βG or HB65-βG. Serum and ascites samples were withdrawn immediately before (0 hr) and 2, 4, 24, 48 and 72 hr after conjugate administration. Serum was collected by centrifugation of whole blood, whereas ascites samples were centrifuged at 200 *g* for 5 min to separate tumor cells from ascites fluid. Pelleted cells were washed once with PBS before resuspension in 1 ml PBS. AS-30D tumor cells, easily distinguishable from the smaller lymphocytes, were counted in a hemacytometer. One hundred microliter samples of serum, ascites fluid, ascites cells or conjugate standard (RH1-βG or HB65-βG) were serially diluted in PBS and added to tubes containing 300 µl reaction buffer (100 mM acetic acid, 50 mM bis-Tris, 50 mM triethanol amine, pH adjusted to 7 with NaOH). Fifty microliters of 32 mM PNPG were added, and tubes were incubated at 37°C for 30 min. Samples were clarified by the addition of 500 µl 0.5 M trichloroacetic acid for 30 min on ice followed by centrifugation at 15,000 *g* for 10 min. Duplicate 70 µl samples from each tube were transferred to 96-well microtiter plates containing 100 µl water and 25 µl 1 N NaOH in each well. Absorbance of wells at 405 nm was determined after 15 min in a Molecular Devices (Menlo Park, CA) microplate reader. Conjugate concentrations in serum, ascites fluid or ascites cells were determined by comparison with the absorbance values of the respective conjugate standards.

Immunofluorescence

Three rats were i.p.-injected on days 1 and 2 with 1.5×10^7 AS-30D cells. On day 6, one rat (rat 1) was i.p.-injected with PBS and 2 rats (rats 2 and 3) were i.p.-injected with 500 µg RH1-βG. Ascites samples were withdrawn from each rat 2 hr later, and AS-30D cells were recovered as described above. One hundred microliter aliquots of 5×10^6 AS-30D cells were incubated on ice with PBS (rats 2 and 3) or various concentrations of RH1-βG (rat 1) for 1 hr. After washing twice with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 1 hr or serially incubated with anti-βG rabbit serum and FITC-conjugated goat anti-rabbit IgG, IgA and IgM. Cells were washed twice with PBS, and the surface immunofluorescence of 10,000 cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Mean fluorescent intensities were estimated with Cell Quest software (Becton Dickinson).

To determine whether the antigen recognized by MAb RH1 was down-regulated by binding of RH1-βG to AS-30D ascites cells *in vivo*, 3 SD rats were i.p.-injected with 1.5×10^7 cells on days 1 and 2. Rats 2 and 3 were i.p.-injected with 700 µg RH1–βG on day 7, and ascites samples were withdrawn from the peritoneal cavity of each rat 2 hr later. Each ascites sample was centrifuged at 200 *g* for 5 min, washed once with cold PBS, resuspended in 200 µl cold PBS and divided into 2 equal 100 µl fractions. One hundred microliters of RH1–βG (200 µg/ml) were added to one fraction and PBS to the other fraction for 1 hr at 4°C. Cells were then washed twice with PBS before addition of FITC-conjugated goat anti-mouse IgG (1:200) for 1 hr at 4°C. Cells were washed twice with PBS, and the surface immunofluorescence of 10,000 cells was measured with a FACSCalibur flow cytometer.

Therapy of hepatoma ascites

Forty-six SD rats weighing 250–300 g were i.p.-injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 6, groups of 8 rats (6 rats for RH1–βG treatment) received i.p. injections of the following compounds: control group, PBS followed 2 hr later with

DMSO/PG (1:5 vol/vol); BHAMG group, PBS followed 2 hr later with 30 mg/kg BHAMG in PBS; pHAM group, PBS followed 2 hr later with 5 mg/kg pHAM in DMSO/PG (1:5 vol/vol); RH1–βG group, PBS followed 2 hr later with 500 μg RH1–βG; 1× treatment group, 500 μg RH1–βG followed 2 hr later with 30 mg/kg BHAMG in PBS; and 3× treatment group, 500 μg RH1–βG followed 2, 3 and 4 hr later with 10 mg/kg BHAMG in PBS.

In a second *in vivo* experiment, 18 SD rats weighing 250–300 g were i.p.-injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 6, groups of 6 rats received i.p. injections of the following compounds: control group, PBS followed 4, 5 and 6 hr later with PBS; RH1–βG treatment group, 500 μg RH1–βG followed 4, 5 and 6 hr later with 10 mg/kg BHAMG in PBS; and HB65–βG treatment group, 500 μg HB65–βG followed 4, 5 and 6 hr later with 10 mg/kg BHAMG in PBS.

Blood samples were collected periodically from each rat for enumeration of platelets, lymphocytes and red blood cells as described above. Animal weights were monitored throughout treatment. Rat survival was followed until only tumor-free animals remained.

Protection from tumor rechallenge

Rats that survived to 100 days after combination treatment with RH1–βG and BHAMG were i.p.-injected with 10^7 AS-30D cells on days 101 and 102 to determine whether protective immunity had been induced by successful tumor therapy. A group of age-matched, untreated rats, maintained in adjacent cages throughout the *in vivo* therapy experiment, were likewise i.p.-injected with 10^7 AS-30D cells on days 1 and 2. Survival was monitored for 100 days.

Statistical analysis

The statistical significance of differences between mean values, and mean survival times (MST) was estimated with the shareware program Schoolstat (White Ant Occasional Publishing, West Melbourne, Australia) using the independent *t*-test for unequal variances. For experiments with long-term survivors, the last day of observation (day 100) was employed for calculation of MST.

RESULTS

Acute toxicity of pHAM and BHAMG

A single i.p. injection of up to 150 mg/kg BHAMG was not lethal, whereas 1 of 4 rats that received 7.5 mg/kg pHAM and 2 of 3 rats injected with 10 mg/kg pHAM died within a week (Table I). All rats that received 20 mg/kg pHAM died within 3 days. The DMSO/PPG vehicle employed to dissolve pHAM did not cause animal mortality (Table I). These results indicate that BHAMG was at least 20 times (w/w) less toxic than pHAM to rats based on animal survival.

Distribution of βG and UDPGT in SD rats

Activities of βG, which may activate glucuronide prodrugs *in vivo*, and UDPGT, which can reverse this reaction by transferring a glucuronide group from UDP-glucuronic acid to hydroxy groups of aglycones, were measured in tissues isolated from SD rats (Fig. 1). βG activities were highest in the liver and spleen, whereas high UDPGT activities were present in the liver and kidney. βG activity was not detected in serum. The activity of UDPGT was similar or higher than βG in most organs, with the notable exceptions of the spleen and colon, in which βG activity significantly ($p < 0.05$ and $p < 0.01$, respectively) exceeded UDPGT activity. βG activity (10.1 ± 0.6 μmol/g/hr) was also slightly higher than UDPGT activity (7.6 ± 0.1 μmol/g/hr) in AS-30D cells, but both enzyme activities were lower than that in the liver. These results suggest that the spleen and colon could be major sites of endogenous activation of glucuronide prodrugs.

TABLE I – LETHAL TOXICITY OF pHAM AND BHAMG

pHAM (mg/kg)	MST[1]	Deaths/total	BHAMG (mg/kg)	MST	Deaths/total
0	>45	0/4	30	>45	0/4
5	>45	0/5	50	>45	0/4
7.5	35	1/4	60	>45	0/4
10	19	2/3	100	>45	0/4
20	2.5	5/5	150	>45	0/4

[1]Mean survival times (MST) of SD rats after i.p. injection of BHAMG or pHAM.

Organ pathology of drug-treated animals

Figure 2 compares the morphologies of the colon, liver, lungs, kidney and spleen of naive rats with rats that received an i.p. injection of 120 mg/kg BHAMG 5 days earlier. The colon of BHAMG-treated rats exhibited normal mucosal lining and structure (Fig. 2*b*). The liver exhibited normal hepatocellular lining without evident cholestasis or cellular infiltration over the portal area (Fig. 2*d*). Lung tissue had normal pulmonary interstitium without alveolar lesions (Fig. 2*f*). Kidney sections did not exhibit evident glomerulosclerosis or tubular necrosis (Fig. 2*h*). Spleen sections had normal red and white pulp configurations with no evidence of mass infiltration (Fig. 2*j*). All tissues recovered from rats 2 or 9 days after BHAMG treatment also appeared normal (data not shown), indicating that BHAMG damage to organs was minimal.

Sublethal toxicity of drugs to rats

Injection of 30 or 60 mg/kg BHAMG i.p. did not significantly affect the weight of SD rats compared to control animals, whereas a single dose of 7.5 mg/kg pHAM resulted in significant weight loss between 2 and 25 days after drug administration; maximum weight loss (−27%) occurred 7 days after pHAM injection (Fig. 3*a*). BHAMG was also less toxic than pHAM to blood cells. Injection of 7.5 mg/kg pHAM i.p. resulted in significant ($p < 0.05$) reduction of peripheral lymphocytes 2 days after drug administration (Fig. 3*b*). The WBC nadir ($1{,}200 \pm 460$ cells/mm^3), corresponding to a 94% reduction in lymphocyte levels compared to rats injected with vehicle alone, was reached 5 days after i.p. injection of 7.5 mg/kg pHAM. In contrast, the number of circulating lymphocytes in rats treated with 30 or 60 mg/kg BHAMG did not differ significantly from that in control rats (Fig. 3*b*). RBC toxicity also was induced by pHAM, though only after day 19, while 30 or 60 mg/kg BHAMG did not produce RBC toxicity (Fig. 3*c*). Similar results were found for platelets in which treatment with 7.5 mg/kg pHAM significantly ($p < 0.05$) reduced platelet numbers after 2 days, whereas injection of 30 or 60 mg/kg BHAMG did not alter platelet

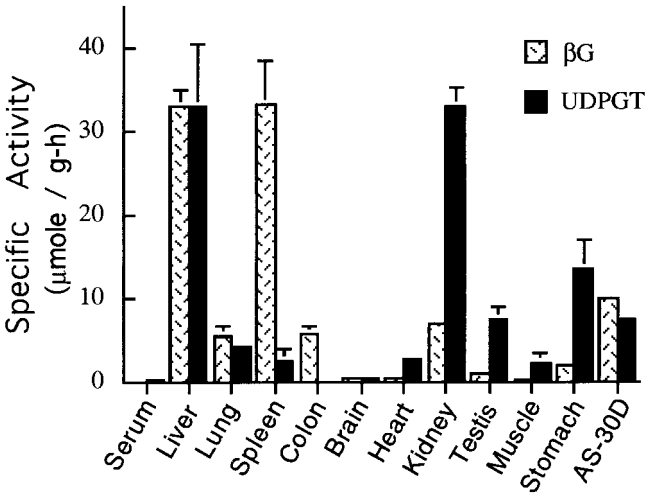


FIGURE 1 – Activities of glucuronide-metabolizing enzymes in SD rats. The activities of UDPGT and βG in tissue homogenates prepared from 3 SD rats were measured. Bars: SE of the mean.

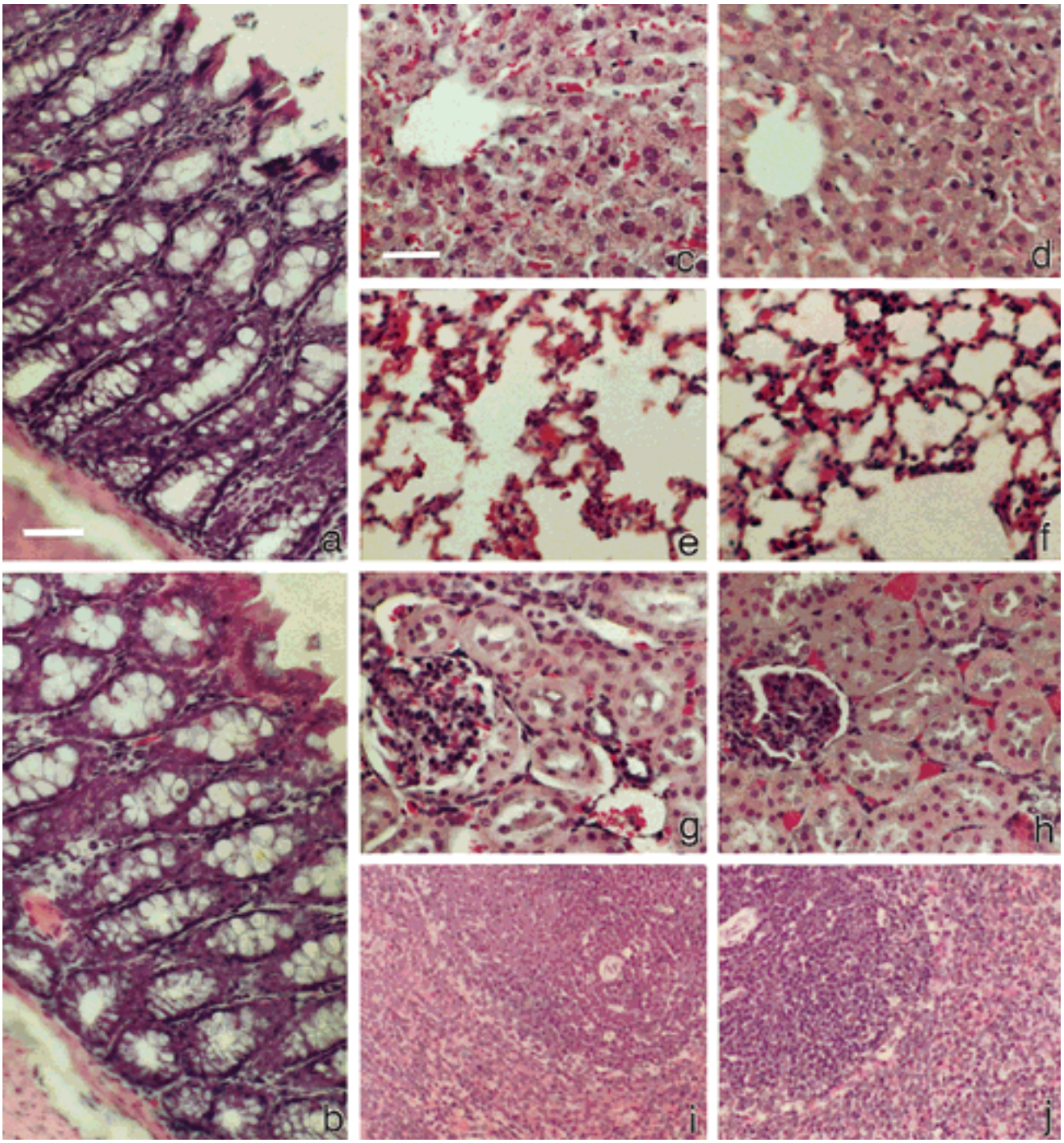


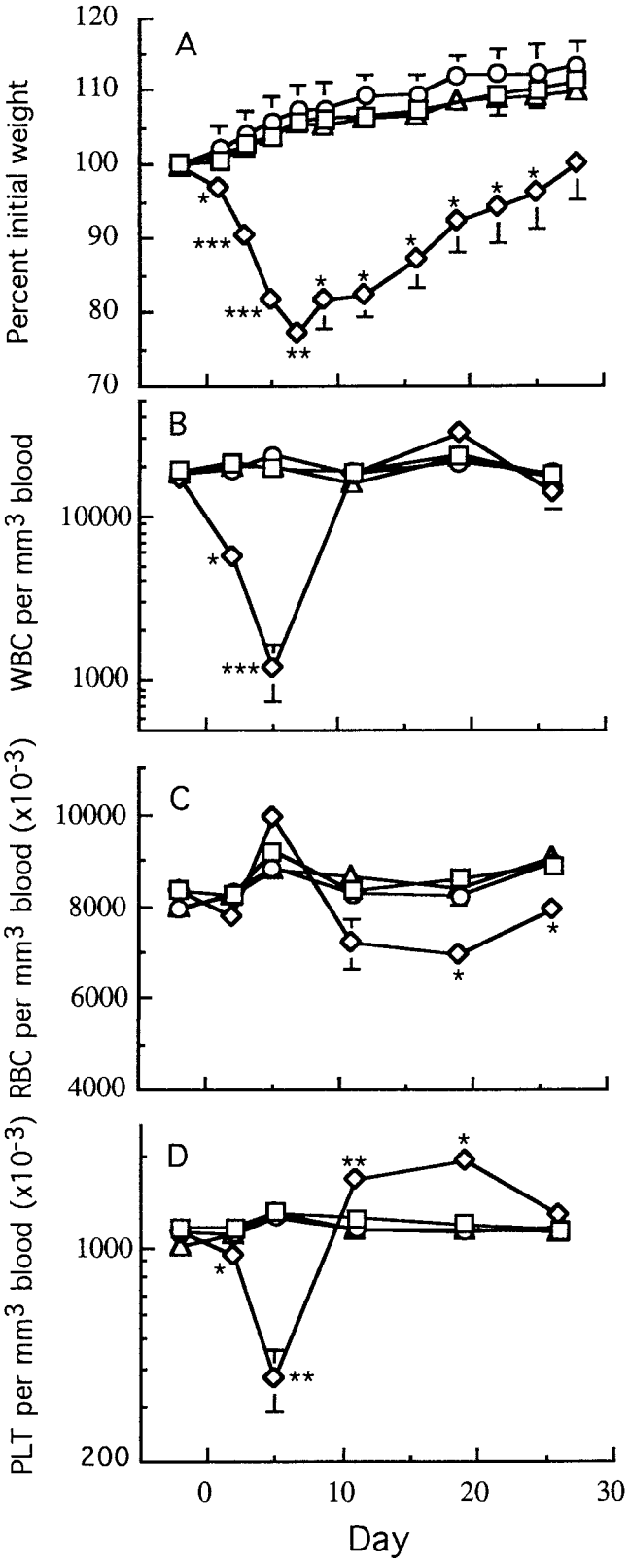
Figure 2 – Organ pathology after BHAMG administration. Tissue sections of organs recovered from rats 5 days after i.p. injection of PBS *(a, c, e, g, i)* or 120 mg/kg BHAMG *(b, d, f, h, j)* were stained with hematoxylin and eosin. *(a, b)* colon, *(c, d)* liver, *(e, f)* lungs, *(g, h)* kidneys and *(i, j)* spleen. Bar: 100 μm.

numbers (Fig. 3*d*). Interestingly, the number of platelets in rats treated with pHAM recovered by day 11 to levels that were significantly ($p < 0.005$) higher than control group levels. Injection of 30 or 60 mg/kg BHAMG did not increase the serum levels of γ-glutamyl transferase above background levels, indicating that the liver was not damaged (data not shown). Taken together, these results indicate that administration of 30 or 60 mg/kg BHAMG did not cause appreciable toxicity to rats, whereas obvious toxicity was observed after a single dose of 7.5 mg/kg pHAM.

Immunoconjugates

Recombinant βG was linked to MAb RH1 and the control MAb HB65 by thioether bonds to form RH1–βG and HB65–βG. Both antibody conjugates retained similar βG activities after conjugation

(Fig. 4*a*). RH1–βG possessed both antigen-binding and enzymatic activities, as shown by the ability of this conjugate to bind to AS-30D cells and to convert PNPG to PNP (Fig. 4*b*). RH1–βG binding to AS-30D cells was specific, as shown by the lack of HB65–βG binding to AS-30D cells (Fig. 4*b*), the ability of MAb RH1 F(ab′)$_2$ fragments to block RH1-βG binding to AS-30D cells (Fig. 4*b*) and the lack of RH1-βG binding to antigen-negative muscle cells (Fig. 4*c*).



Tumor localization of RH1–βG

Injection of 500 μg RH1–βG i.p. in SD rats bearing established hepatoma ascites resulted in rapid tumor localization, with accumulation of 54 ± 4.9 μg RH1–βG per 10^9 AS-30D cells after 2 hr (Fig. 5*a*). RH1–βG activity at AS-30D cells decreased with first-order kinetics ($r^2 = 0.995$) with a half-life of 8.5 hr. RH1–βG was not detected in serum at any time, whereas a maximum of 0.53 ± 0.31 μg/ml RH1–βG was measured in ascites fluid 2 hr after administration of conjugate (Fig. 5*b*,*c*). RH1–βG localization at AS-30D cells was antibody-mediated, as shown by the significantly lower accumulation of HB65–βG at AS-30D cells throughout the observation period (maximum 2.74 ± 0.95 μg per 10^9 AS-30D cells after 4 hr). HB65–βG concentrations in ascites fluid (Fig. 5*b*) and serum (Fig. 5*c*), in contrast, were significantly higher than RH1–βG at all times.

RH1–βG immunofluorescence

AS-30D cells recovered from ascites and stained *in vitro* with RH1–βG conjugate and FITC-conjugated goat anti-mouse IgG exhibited surface fluorescence that was dependent on RH1–βG concentration (Fig. 6*a*). Antigen sites on AS-30D cells could be saturated by *in vitro* incubation of 5×10^6 cells with approx. 100 μg/ml RH1–βG (Fig. 6*b*). AS-30D cells recovered from the ascites of rats 2 hr after injection of 500 μg RH1–βG exhibited immunofluorescence intensities ranging over 3 orders of magnitude after surface-bound conjugate was probed with FITC-conjugated goat anti-mouse IgG (Fig. 6*c*,*d*). This result shows that surface antigen on AS-30D cells was not saturated after i.p. injection of 500 μg RH1–βG and that conjugate binding to AS-30D cells was heterogeneous, with some tumor cells binding almost no conjugate.

In a separate experiment, *in vitro* addition of RH1–βG to ascites cells pre-targeted with RH1–βG *in vivo* resulted in increased surface immunofluorescence (Fig. 6*f*) similar to non-targeted ascites cells incubated with RH1–βG *in vitro* (Fig. 6*e*), showing that AS-30D surface antigen was not down-regulated by RH1–βG *in vivo*. The maximum fluorescence was relatively low due to the large number of AS-30D cells recovered from ascites in this experiment.

AS-30D cells recovered from ascites 2 hr after i.p. injection of 500 μg RH1–βG also were stained with rabbit anti-βG serum and FITC-conjugated goat anti-rabbit IgG, IgA and IgM. βG was present at less than saturating levels but greater than background levels (Fig. 7*a*) on ascites cells 2 hr after i.p. injection of conjugate (Fig. 7*b*,*c*), similar to the results obtained after probing for cell-bound immunoglobulin (Fig. 6*c*,*d*). This result shows that βG was present on the membrane surface of ascites tumor cells 2 hr after i.p. injection of RH1–βG and indicates that the conjugate was stable during this period.

In vivo *therapy of rat hepatoma ascites*

The efficacy of combined treatment of malignant ascites with RH1–βG and BHAMG was examined in SD rats (Fig. 8). Treatment was initiated on day 6 after inoculation of a total of 3×10^7 AS-30D cells to allow development of advanced ascites. All untreated rats (8/8) succumbed to massive ascites by day 14

FIGURE 3 – Toxicity of pHAM and BHAMG in SD rats. Groups of 4 SD rats were injected i.p. on day 0 with DMSO/PG (1:5 vol/vol) (□), 7.5 mg/kg pHAM in DMSO/PG (◇) or 30 (○) or 60 (△) mg/kg BHAMG in PBS. The mean weight of rats *(a)* as well as the number of WBCs *(b)*, RBCs *(c)* and platelets *(d)* in blood samples were measured 2 days before administration of drugs and at the indicated times after drug treatment. Significant differences between treated and control (vehicle alone) groups are shown (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). Bars: SE of the mean.

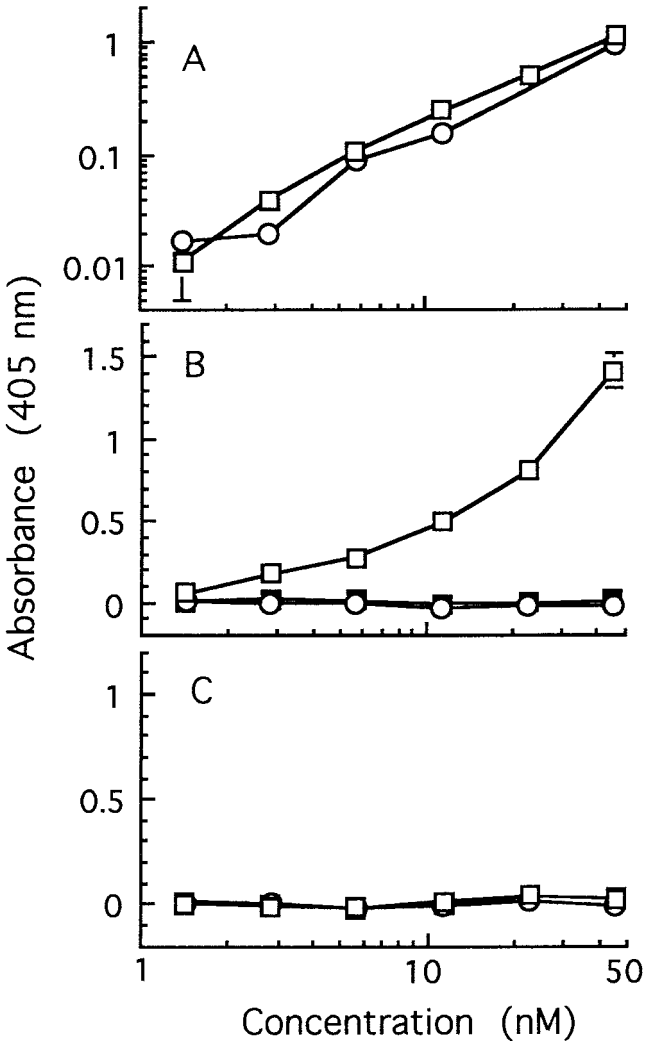


FIGURE 4 – Activity and specificity of conjugates. *(a)* Conversion of PNPG to PNP by RH1–βG (□) and HB65–βG (○) at 37°C, pH 7.0, measured after 30 min at 405 nm. Serial dilutions of RH1–βG (□), HB65–βG (○) or RH1–βG with 1,000 μg/ml RH1 $F(ab)'_2$ fragments (■) were incubated overnight at 4°C in 96-well plates coated with AS-30D *(b)* or NOR 10 *(c)* cells before plates were washed with PBS. Conversion in each well of PNPG to PNP after 1 hr at 37°C, pH 7.0, was monitored at 405 nm. Mean values and SEs of quadruplicate determinations are shown.

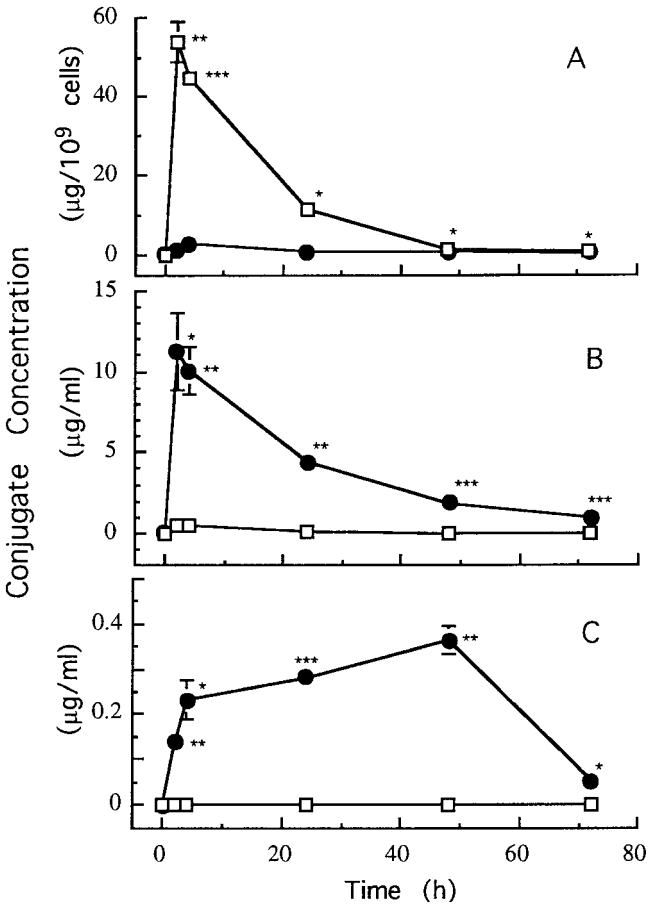


FIGURE 5 – *In vivo* localization of RH1–βG. Groups of 4 SD rats received i.p. injections of 1.5×10^7 AS-30D cells on days 1 and 2. Concentrations of RH1–βG (□) and HB65–βG (●) were determined in *(a)* AS-30D cells recovered from ascites, *(b)* ascites fluid or *(c)* serum taken immediately before i.p. injection of 500 μg RH1–βG or HB65–βG on day 6 and 2, 4, 24, 48 and 72 hr later. Mean conjugate concentrations and standard errors at each time are shown. Significant differences between the concentrations of RH1–βG and HB65–βG are indicated (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

(MST = 11.5 days). In contrast, treatment with 500 μg RH1–βG followed 2 hr later with a single i.p. injection of 30 mg/kg BHAMG significantly ($p < 0.005$) prolonged animal survival (MST > 86 days), with 6 of 8 rats surviving to day 100. Treatment with 3 hourly doses of 10 mg/kg BHAMG starting 2 hr after administration of RH1–βG resulted in 100% (8/8) long-term survivors. Cure of ascites required pre-treatment with RH1–βG since 30 mg/kg BHAMG alone did not extend significantly the life of tumor-bearing rats (MST = 19.6 days). Treatment with 5 mg/kg pHAM, judged to be near the maximal tolerated dose based on toxicity studies, also did not extend significantly the survival of rats (MST = 15.5 days). Treatment with RH1–βG alone did not provide any therapeutic benefit (MST = 10.7 days). All rats that died after day 50 exhibited large solid tumors in the peritoneal region.

Toxicity of in vivo *therapy*

The weight of untreated rats rapidly increased until day 10 and then declined until death (Fig. 9*a*), presumably reflecting accumulation of ascites and fluid in the peritoneal cavity followed by morbidity due to massive ascites. Rats treated with RH1–βG and BHAMG experienced mild weight loss immediately after treatment but began gaining weight by day 17. The initial weight loss could reflect treatment toxicity but also could be due to elimination of ascites and retained fluid. Measurement of circulating blood cells represents a more sensitive measure of drug-induced toxicity by activated BHAMG. Combination treatment with RH1–βG and BHAMG was well tolerated, as shown by the significant ($p < 0.05$) but modest decrease in WBC levels from 19,500 cells/mm^3 before treatment to 12,600 and 10,600 cells/mm^3 after treatment with 30 mg/kg BHAMG or 3 fractionated doses of 10 mg/kg BHAMG, respectively (Fig. 9*b*). The number of WBCs in untreated and BHAMG-treated rats was elevated significantly on days 9 ($p < 0.005$) and 14 ($p < 0.05$) compared to pre-treatment levels (Fig. 9*b*), suggesting that tumor cells induced an immune response, albeit an ineffective one. The number of RBCs in rats treated with RH1–βG and BHAMG was also reduced significantly compared to pre-treatment levels (Fig. 9*c*) but was higher than the number in untreated tumor-bearing rats. Treatment with RH1–βG followed by 30 mg/kg BHAMG produced a small reduction in the number of circulating platelets on day 9, whereas 3 fractionated doses of 10 mg/kg BHAMG administered 2 hr after injection of RH1–βG did not cause platelet toxicity (Fig. 9*d*). pHAM did not produce

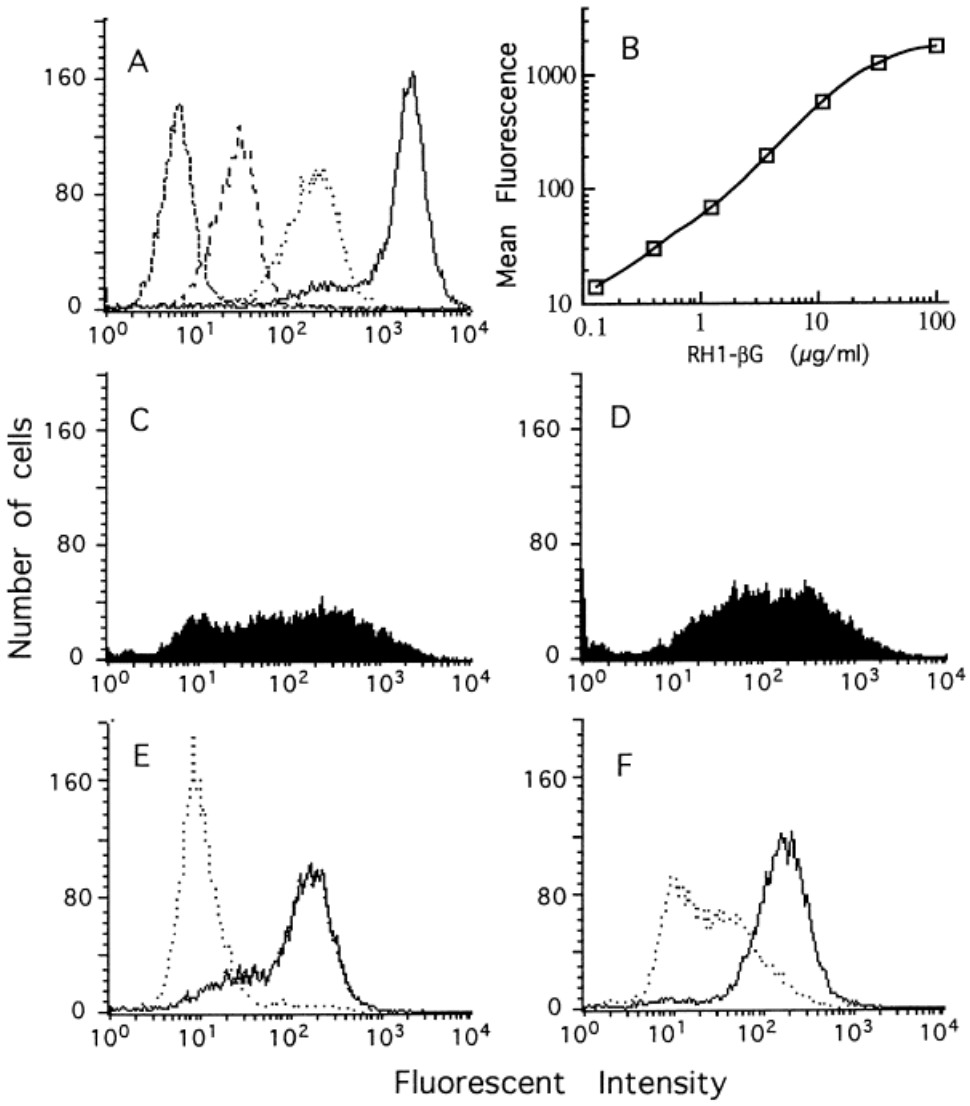


FIGURE 6 – Flow-cytometric determination of RH1–βG at AS-30D ascites cells. *(a)* Ascites cells were incubated with 0 (- - -), 0.4 (— —), 3.7 (·····) or 100 (——) μg/ml RH1βG before staining cells with FITC-conjugated goat anti-mouse IgG. *(b)* Mean fluorescence intensity of ascites cells incubated *in vitro* with various concentrations of RH1–βG before staining with FITC-conjugated goat anti-mouse IgG. *(c, d)* Ascites cells recovered from rats 2 hr after injection of 500 μg RH1–βG were incubated with FITC-conjugated goat anti-mouse IgG. *(e)* Fluorescence of ascites cells incubated with 0 (·····) or 200 (——) μg/ml RH1βG followed by FITC-conjugated goat anti-mouse IgG. (*f*) Ascites cells recovered from rats 2 hr after i.p. injection of 700 μg/ml RH1–βG were incubated with 0 (·····) or 200 (——) μg/ml RH1βG followed by FITC-conjugated goat anti-mouse IgG. The immunofluorescence of 10,000 cells from each sample was determined by flow cytometry.

significant hematological toxicity, indicating that drug toxicity was not responsible for the deaths in the pHAM treatment group.

Treatment specificity

Treatment specificity was examined by comparing the efficacy of combined treatment with BHAMG and RH1–βG or the control antibody conjugate HB65–βG. Combined treatment with 500 μg of either conjugate followed 4, 5 and 6 hr later by i.p. injections of 10 mg/kg BHAMG resulted in the cure of all rats. All untreated tumor-bearing rats, as expected, died with an MST of 17.3 days. Treatment with HB65–βG and BHAMG, however, reduced WBC numbers by 75%, whereas treatment with RH1–βG and BHAMG did not reduce significantly WBC numbers from pre-treatment levels (Fig. 10). Treatment with HB65–βG and BHAMG caused significantly ($p \leq 0.01$) more WBC toxicity than combined treatment with the specific antibody–enzyme conjugate and BHAMG.

Tumor rechallenge of cured rats

Rats that survived to day 100 after treatment with RH1–βG and BHAMG (Fig. 8) were rechallenged with 2×10^7 AS-30D cells. All of the cured rats (14/14) developed protective immunity, as shown by complete rejection of the tumor challenge, whereas all 8 age-matched control rats died within 21 days (MST = 16.9 days) (Table II). In addition, all rats that were cured in the second *in vivo* experiment (Fig. 10) by combined treatment with RH1–βG or HB65–βG and BHAMG were protected from rechallenge with 1.5×10^7 AS-30D cells 60 days after treatment.

DISCUSSION

One concern of employing glucuronide prodrugs for cancer therapy is the possibility that endogenous βG present in various tissues could activate the prodrug and cause non-specific toxicity. Several organs, including the liver, gastrointestinal tract, spleen and

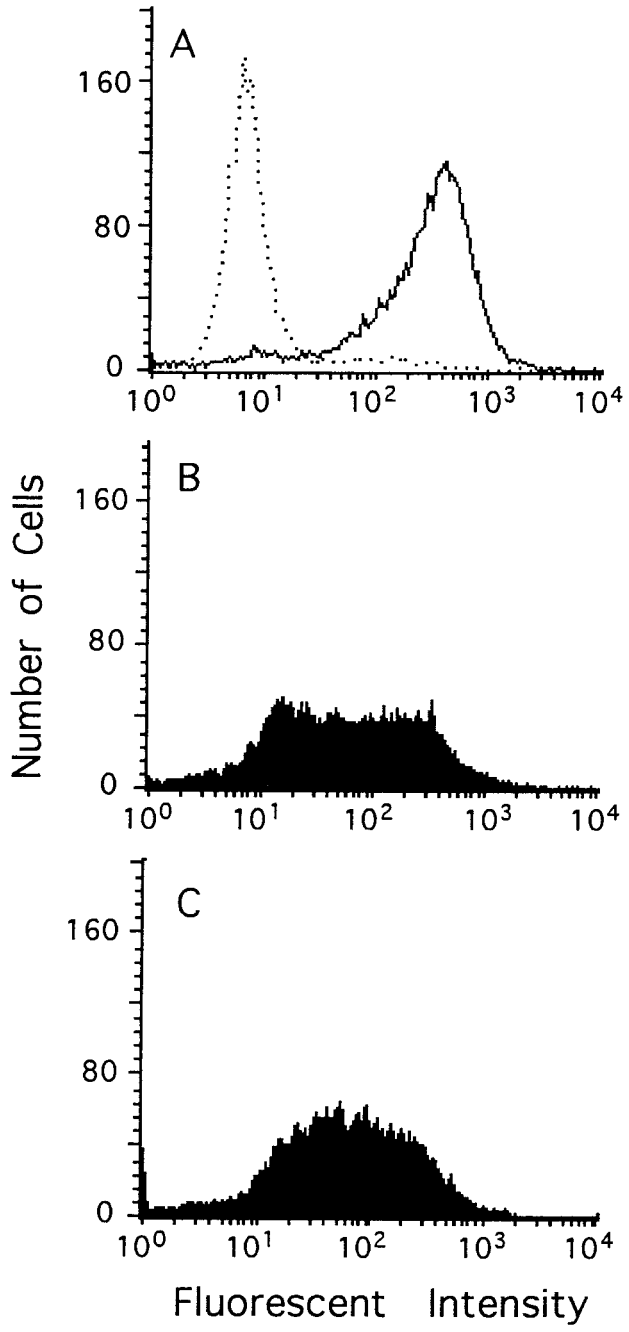


FIGURE 7 – Flow-cytometric determination of βG localization at AS-30D ascites cells. Three rats bearing established AS-30D ascites were i.p.-injected with PBS *(a)* or 500 μg RH1–βG *(b, c)*. Two hours later, ascites cells were recovered and incubated with 0 (·····) or 100 (——) μg/ml RH1–βG *(a)* or PBS *(b, c)* for 1 hr. Ascites cells then were incubated with rabbit anti-βG serum and FITC-conjugated goat anti-rabbit IgG, IgA and IgM before the immunofluorescence of 10,000 cells from each sample was measured by flow cytometry.

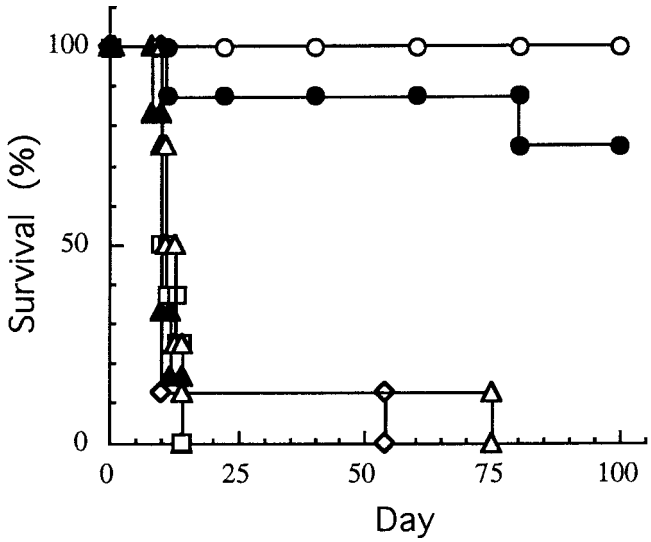


FIGURE 8 – Therapy of malignant ascites. Groups of 8 SD rats bearing established AS-30D ascites were i.p.-injected on day 6 with PBS followed 2 hr later by an injection of DMSO/PG (1:5 vol/vol) (□), PBS followed 2 hr later with 5 mg/kg pHAM in DMSO/PG (◇), PBS followed 2 hr later with 30 mg/kg BHAMG (△), 500 μg RH1–βG followed 2 hr later with PBS (▲), 500 μg RH1–βG followed 2 hr later with 30 mg/kg BHAMG (●) or 500 μg RH1–βG followed 2, 3 and 4 hr later with 10 mg/kg BHAMG (○). Survival of rats was monitored for 100 days.

lungs, contained relatively high activities of βG. Most organs, with the exceptions of the colon and spleen, also expressed comparable activities of UDPGT, a class of enzymes that can reverse the reaction catalyzed by βG. The relatively high level of βG in the gastrointestinal tract was likely due to the presence of bacteria in the colon. Human fecal samples contain 1–2 orders of magnitude less βG activity than do samples from rats (Nolen *et al.*, 1995), suggesting that secretion of βG by gut bacteria may be less important in humans. The morphologies of the colon, spleen, lung, liver, and kidneys after i.p. injection of 120 mg/kg BHAMG, however, were identical to those in control animals, showing that prodrug damage to normal tissues was minimal.

The dose-limiting toxicity of alkylating agents usually is caused by severe suppression of hematological cells. BHAMG, however, caused significantly less toxicity to blood cells than pHAM. Injection of 7.5 mg/kg pHAM i.p. reduced the number of circulating WBCs in rats by 94%, while 60 mg/kg BHAMG did not affect WBC numbers. The low toxicity of BHAMG to blood cells is consistent with the absence of detectable levels of βG in serum. βG activity in human serum is also extremely low (Stahl and Fishman, 1984), suggesting that the hematological toxicity of BHAMG should be minimal in humans.

The low toxicity of BHAMG relative to pHAM most likely can be attributed to the high polarity of the prodrug. Cellular cytotoxicity of nitrogen mustards is believed to be mediated primarily by guanine alkylation at the N-7 position of DNA, leading to DNA cross-linking and prevention of DNA-strand separation during replication (Lawley and Brookes, 1965). β-D-Glucuronides typically favor partition into the aqueous phase and exhibit reduced cellular uptake relative to their aglycones (Haisma *et al.*, 1992), suggesting that the low toxicity of BHAMG is due to hindered cellular entry of the prodrug. The lack of tissue damage after injection of BHAMG is also consistent with this mechanism since βG is located predominantly in lysosomal and microsomal compartments of mammalian tissues, sites relatively inaccessible to polar glucuronide prodrugs.

RH1–βG rapidly localized at AS-30D ascites cells in rats with accumulation of 54 μg RH1–βG per 10^9 AS-30D cells 2 hr after i.p. injection of RH1–βG (Fig. 5*a*). Although this corresponds to binding of approx. 150,000 molecules of RH1–βG per cell, antigen sites on AS-30D cells were not saturated with conjugate, as shown by immunofluorescence analysis of individual tumor cells (Fig. 6). The low concentrations of RH1–βG in ascites fluid at all times examined supports the notion that RH1–βG did not saturate antigen sites on AS-30D cells and indicates that most of the

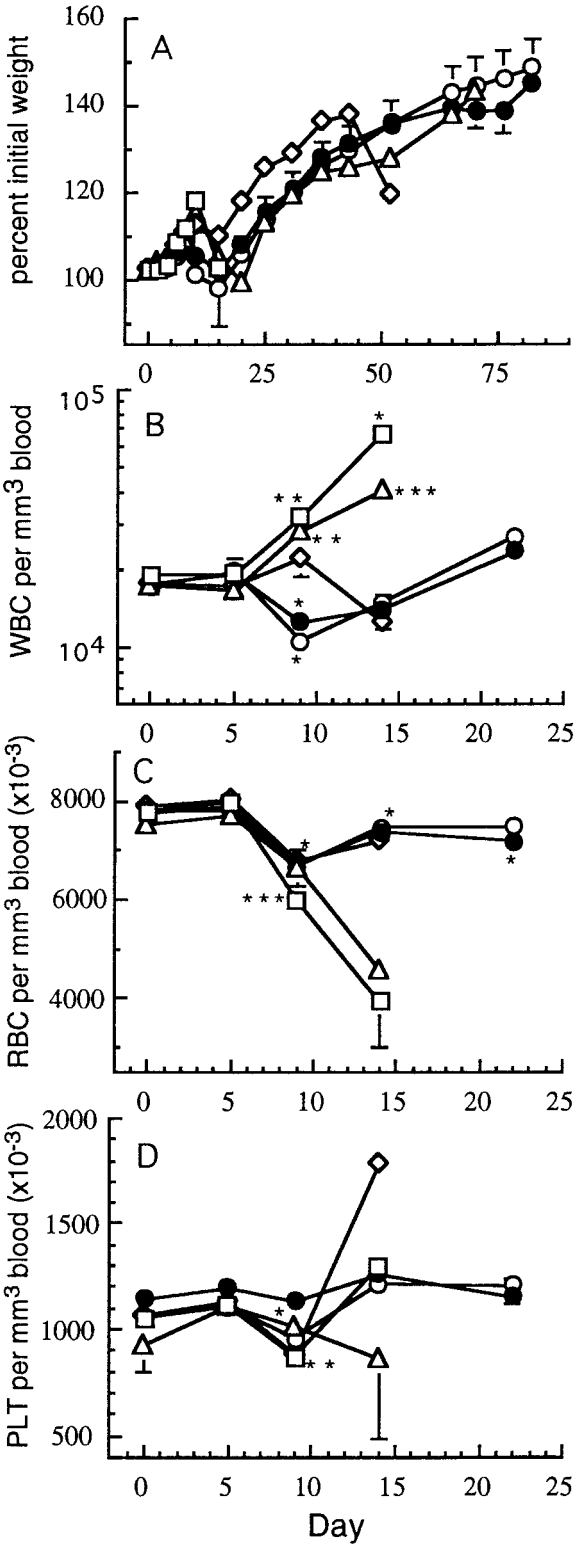


FIGURE 9 – Toxicity of prodrug treatment. Mean relative weights (% of initial weight) *(a)* and numbers of WBCs *(b)*, RBCs *(c)* and platelets *(d)* of groups of 8 SD rats treated as described in the legend to Figure 8 are shown. Significant differences between mean values before treatment (day 5) and after treatment are indicated: $*p \leq 0.05$, $**p \leq 0.005$, $***p \leq 0.0005$. Data for control (□), BHAMG (△) and pHAM (◇) groups on day 14 represent values from 2, 2 and 1 rat, respectively, due to animal mortality. Bars: SE of the mean.

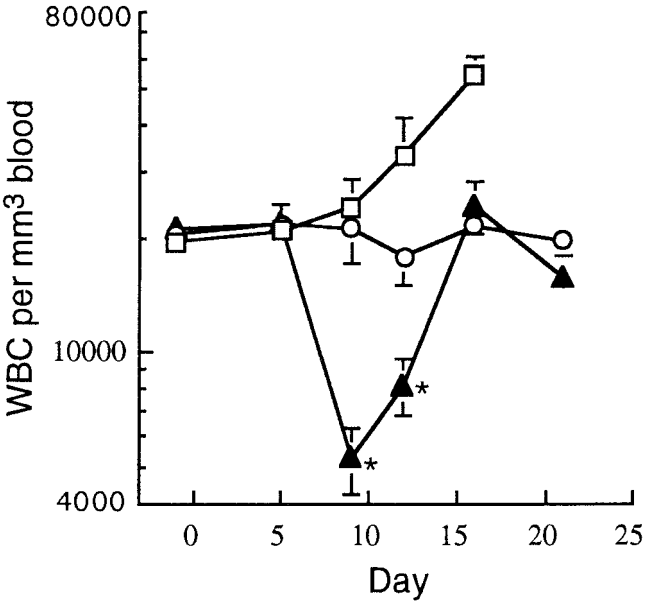


FIGURE 10 – Specificity of prodrug treatment. Mean numbers of WBCs for groups of 6 SD rats bearing established AS-30D ascites are shown after i.p. injection on day 6 with PBS (□); 500 µg RH1–βG followed 4, 5 and 6 hr later with 10 mg/kg BHAMG (○); or 500 µg HB65–βG followed 4, 5 and 6 hr later with 10 mg/kg BHAMG (▲). Significant differences between mean values of RH1–βG- and HB65–βG-treated rats are indicated: $*p \leq 0.01$. Bars: SE of the mean.

TABLE II – PROTECTIVE IMMUNITY OF CURED RATS[1]

Group	Deaths/total	MST
Control	8.8	17.3 ± 0.9
RH1–βG + BHAMG (1×)	0/6	>100
RH1–βG + BHAMG (3×)	0/8	>100

[1]Rats surviving to day 100 after prodrug treatment with RH1–βG and a single dose (1×) or 3 fractionated doses (3×) of BHAMG and a group of 8 age-matched naive rats (control) were i.p.-injected with 1×10^7 AS-30D cells on days 101 and 102. Survival of rats was monitored for 100 days.

injected dose of RH1–βG specifically accumulated at ascites tumor cells. The low binding of the control antibody conjugate HB65–βG to ascites cells confirmed that RH1–βG binding was antibody-mediated. RH1–βG activity at AS-30D ascites cells decreased, with a half-life of 8.5 hr, about 2.5 times faster than would be expected by simple dilution from replicating AS-30D cells (doubling time = 21 hr [Wang *et al.*, 1992]). Although RH1–βG did not internalize into AS-30D cells over a 20 hr period *in vitro* (data not shown), we cannot rule out the possibility that slow internalization or accelerated *in vivo* internalization of the conjugate occurred. Conjugate activity also could decrease due to *in vivo* degradation or to inhibition of βG activity by serum proteins (Haisma *et al.*, 1992), though we have shown that βG is stable in both rodent and human serum for 24 hr at 37°C (Cheng *et al.*, 1997). Targeting studies, however, clearly demonstrated that significant amounts of active conjugate were present on the surface of AS-30D ascites cells 2 hr after administration of RH1–βG. Localization of βG to the surface of tumor cells is expected to be important because it is likely that hydrophilic glucuronide prodrugs cannot freely diffuse across the lipid bilayer.

In vivo therapy of rat hepatoma ascites was examined by first administering RH1–βG and then treating with BHAMG. Treatment with RH1–βG followed 2 hr later with injection of 30 mg/kg BHAMG cured 6 of 8 rats bearing large hepatoma ascites ($>2 \times 10^9$ cells), whereas administration of 3 fractionated doses of

10 mg/kg BHAMG resulted in the cure of 100% of rats (8/8). Although 30 mg/kg BHAMG was less than the maximum tolerated dose (>60 mg/kg), mild transient hematological toxicity was observed, suggesting that activated drug entered the circulation. No significant hematological toxicity, however, was found when the prodrug was administered starting 4 hr after injection of RH1–βG (Fig. 10). The superior efficacy achieved by administering fractionated doses of BHAMG indicates that prolonged exposure of tumor cells to activated drug is advantageous and that continuous infusion might be the preferred method of prodrug administration.

Treatment of rats with either BHAMG or RH1–βG did not extend significantly survival beyond that of untreated rats, showing that successful therapy required combination treatment with RH1–βG and prodrug. Surprisingly, pHAM was also ineffective. The lack of pHAM efficacy cannot be attributed to a suboptimal dose since 5 mg/kg was judged to be the maximum tolerated dose in SD rats. Several possibilities exist for the lack of pHAM efficacy given the excellent efficacy of the targeted activation of BHAMG. First, the administered dose of BHAMG was more than 2-fold greater than the pHAM dose on a molar basis, suggesting that higher concentrations of active drug may have been produced at tumor cells by activation of BHAMG. Second, alkylating agents can be inactivated by hydrolysis (Wilman *et al.*, 1995). BHAMG is hydrolyzed 3 times slower than pHAM ($\tau_{1/2} = 7$ min) in aqueous solutions (data not shown). The greater stability of BHAMG could have prolonged exposure of tumor cells to activated prodrug. Third, hydrophobic drugs, including alkylating agents, readily bind to proteins *in vivo* (Chang *et al.*, 1978). Glucuronide conjugates, in contrast, often exhibit markedly reduced protein binding (Mulder *et al.*, 1985), suggesting that BHAMG binding to proteins could be reduced compared with pHAM. Activation of BHAMG near tumor cells could allow increased uptake of pHAM before significant protein binding occurred. Finally, localization of RH1–βG at tumor cells may have augmented an anti-tumor immune response against AS-30D cells, though this possibility seems unlikely given the poor efficacy of RH1–βG treatment.

Combined treatment of ascites with BHAMG and HB65–βG also cured rats, indicating that the generation of pHAM by the high concentration of conjugate present in the ascites fluid during prodrug administration could induce tumor regression. This result and the observation that RH1–βG binding to tumor cells was extremely heterogeneous (Fig. 6*c*,*d*) suggest that activated BHAMG can produce strong bystander killing of tumor cells. Treatment with RH1–βG and BHAMG, however, was superior based on significantly lower hematological toxicity, corresponding to improved treatment specificity. The higher toxicity observed after combined treatment with HB65–βG and BHAMG likely can be attributed to systemic activation of prodrug in the circulation. Further studies to examine the dose-response of conjugates and prodrug may reveal conditions in which only the specific conjugate and BHAMG are effective.

An important finding of our study was that successful treatment of hepatoma ascites by targeted activation of BHAMG induced complete protective immunity to subsequent challenge with a lethal dose of AS-30D tumor cells. The significant increase in circulating lymphocytes in untreated tumor-bearing rats (Fig. 9*b*) suggests that an immune response against AS-30D cells normally develops but that this response is inadequate to reject completely the large tumor load present in ascites. Killing of large numbers of tumor cells by the targeted activation of BHAMG may allow sufficient time for immune-mediated control of tumor growth as well as provide additional antigen for professional antigen-presenting cells. Antineoplastic drugs also have been shown, under some conditions, to enhance T cell–mediated immunity in animal models (Morikawa *et al.*, 1985; Awwad and North, 1989) as well as in patients (Berd and Mastrangelo, 1987). These results raise the possibility that generation of pHAM at the tumor site may enhance anti-tumor immunity. Current studies are under way to define the mechanism of anti-tumor immunity after combined treatment with RH1–βG and BHAMG as well as to determine whether generation of pHAM at tumor cells can potentiate anti-tumor immunity.

The development of protective immunity after prodrug treatment has several implications. First, rejection of a large tumor challenge after successful treatment of the primary malignancy with RH1–βG and BHAMG shows that prodrug treatment did not prevent the generation of an anti-tumor immune response. Second, it may not be necessary to kill every tumor cell with activated prodrug since immune cells may produce bystander killing of tumors. Finally, reduction of tumor load by targeted activation of prodrugs followed by immunotherapy may provide a rational approach for improving the efficacy of cancer therapy.

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