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POTENTIATION OF ANTITUMOR IMMUNITY BY ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY

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Antibody-directed enzyme prodrug therapy (ADEPT) has displayed antitumor activity in animal models and clinical trials. We examined whether antitumor immunity is generated during ADEPT by employing an immunoenzyme composed of the monoclonal antibody (MAb) RH1 conjugated to β-glucuronidase to target rat AS-30D hepatocellular carcinoma tumors. A glucuronide prodrug of p-hydroxyaniline mustard was used to treat malignant ascites after immunoenzyme localization at the cancer cells. ADEPT cured more than 96% of Sprague-Dawley rats bearing advanced malignant ascites, and all cured rats were protected from a lethal challenge of AS-30D cells. Immunization with radiation-killed AS-30D cells or AS-30D cells coated with immunoenzyme did not provide tumor protection. Likewise, *ex vivo* treatment of tumor cells by ADEPT before injection into rats did not protect against a tumor challenge. AS-30D and N1-S1 hepatocellular carcinoma cells but not unrelated syngeneic tumor cells were lysed by peritoneal exudate cells isolated from ADEPT-cured rats. Depletion of $CD8^+$ but not $CD4^+$ T cells or natural killer (NK) cells reduced the cytolytic activity of peritoneal lymphocytes. ADEPT did not cure tumor-bearing rats depleted of $CD4^+$ and $CD8^+$ T cells even though it was curative when given 7 days after tumor transplantation in rats with an intact immune system, indicating that ADEPT can synergize with host immunity to increase therapeutic efficacy. These results have important implications for the clinical application of ADEPT.
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Key words: *ADEPT; prodrug; immunoenzyme; hepatocellular carcinoma; immunotherapy*

Chemotherapy is an important treatment modality for advanced cancers. Methods that increase the therapeutic index of chemotherapy can potentially improve treatment efficacy and reduce the side effects associated with systemic drug delivery. Antibody-directed enzyme prodrug therapy (ADEPT) was developed to achieve these goals.[1,2] In this treatment strategy, a conjugate formed between an antibody and an enzyme (immunoenzyme) is first administered. After the immunoenzyme accumulates at tumor cells, a relatively nontoxic prodrug is systemically administered to allow selective enzymatic activation of the prodrug at the tumor cells. ADEPT has demonstrated advantages for tumor therapy in animal models including high accumulation of drug at solid tumors,[3] bystander killing of antigen-negative tumor cells[4] and improved efficacy compared with conventional chemotherapy.[5] Promising antitumor activity has been demonstrated in a phase I clinical trial.[6]

Tumor cells may escape destruction by ADEPT due to physiologic barriers that prevent contact of immunoenzyme with tumor cells, loss of tumor-associated antigen on the tumor surface or the development of drug resistance. Induction of antitumor immunity and subsequent immune-mediated killing of inaccessible or drug-resistant tumor cells could greatly enhance the therapeutic potential of ADEPT. Antitumor immunity in ADEPT, however, has remained unexplored because immunodeficient mice have been employed in most studies.[7] In the present investigation, we employed a rat model of malignant hepatocellular carcinoma ascites to investigate the relationship between ADEPT and antitumor immunity. We show that ADEPT can synergize with the immune system to provide increased therapeutic efficacy.

MATERIAL AND METHODS

Reagents

BHAMG and pHAM were synthesized as described.[8] Polyinosinic-polycytidylic acid and egg ovalbumin were from Sigma (St. Louis, MO). Recombinant βG was produced as described.[9] Sprague-Dawley rats and BALB/c mice were obtained from and maintained in the animal room of the Institute of Biomedical Science, Academia Sinica. Animal experiments were performed in accordance with institute guidelines.

Cells

AS-30D rat hepatocellular carcinoma cells[10] were generously provided by Dr. J.P. Chang (Institute of Zoology, Academia Sinica, Taipei, Taiwan). AS-30D cells were passaged *in vivo* as ascites in SD rats. NMU mammary adenocarcinoma (CRL-1773), N1-S1 hepatocellular carcinoma (CRL-1604), C6 glioma (CCL-107) and YAC-1 lymphoma (TIB-160) cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

Antibodies

The MAb RH1 is a murine IgG2a MAb that binds a 32 kDa antigen expressed on the surface of AS-30D cells.[11] The MAb RH1 has previously been employed for ADEPT in SD rats[12] and in scid mice.[9] OX-8, OX-35, OX-38 and 3.2.3 hybridomas were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Hybridomas were recloned by limiting dilution and secretion of antibodies was verified by ELISA. Hybridoma ascites were produced in pristane-primed mice. The MAb RH1 was purified from ascites by protein-A affinity chromatography. W3/25-FITC (anti-CD4), OX8-FITC (anti-CD8), OX19-PE (anti-CD5) and 3.2.3-FITC (anti-NK) were purchased from Serotec

Abbreviations: 5-FC, 5-fluorocytosine; ADEPT, antibody-directed enzyme prodrug therapy; APC, antigen-presenting cells; βG, *E. coli*-derived β-glucuronidase; BHAMG, tetra n-butyl ammonium salt of the glucuronide of p-hydroxyaniline mustard; CD, cytosine deaminase; CTL, cytotoxic T lymphocyte; E:T, effector/target ratio; FITC, fluorescein isothiocyanate; GCV, ganciclovir; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-α, interferon-α; IL, interleukin; MAb, monoclonal antibody; NK, natural killer (cell); OVA, ovalbumin; PEC, peritoneal exudate cells; pHAM, p-hydroxyaniline mustard; RBC, red blood cell; RH1-βG, conjugate of MAb RH1 with β-glucuronidase; SD, Sprague-Dawley; tk, thymidine kinase.

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(Oxford, UK). Goat anti-rat Ig and FITC-conjugated goat anti-mouse Ig were from Organon Teknika (Turnhout, Belgium).

Antibody-directed enzyme prodrug therapy

Recombinant βG was covalently linked to the MAb RH1 as described to create RH1-βG.[13] SD rats weighing 250–350 g were i.p. injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 7, rats were i.p. injected with PBS or 300 μg RH1-βG followed 2, 3 and 4 hr later with 7.5 mg/kg BHAMG in PBS. Age-matched naive controls and long-term survivors were challenged after various periods with 10^7 live AS-30D cells (i.p.) on 2 sequential days. Rats surviving for 60 days without formation of tumors were considered cured. The specificity of ADEPT was examined in groups of 4 SD rats by i.p. injection of 1.5×10^7 AS-30D cells on days 1 and 2. On day 7, rats were i.p. injected with PBS, 700 pg-mol RH1, 700 pg-mol RH1 and 700 pg-mol βG or 700 pg-mol RH1-βG followed 2, 3 and 4 hr later with PBS, 7.5 mg/kg BHAMG in PBS or a single injection of 5 mg/kg pHAM.

Tumor immunization and challenge

Groups of 8 SD rats were i.p. injected on day 1 with doses of 10^7, 10^8 or 10^9 lethally irradiated (40 cGy) AS-30D cells. Control rats ($n = 6$) were injected with PBS. All rats were challenged with i.p. injections of 10^7 live AS-30D cells on days 100 and 101. In a separate experiment, groups of 6 SD rats were i.p. injected on day 0 with 10^8 lethally irradiated AS-30D cells or 10^8 irradiated AS-30D cells that had been treated with 300 μg RH1-βG. Another group of rats was i.p. injected with 1.5×10^7 live AS-30D cells on days −5 and −4 and then treated by ADEPT on day 1. All rats were challenged with 10^7 live AS-30D cells on days 50 and 51.

Ex vivo *antibody-directed enzyme prodrug therapy*

Forty-eight SD rats were divided into 6 groups of 8 rats. The first group was i.p. injected with 1.5×10^7 live AS-30D cells on days 1 and 2 and then treated by the standard ADEPT protocol on day 7. Then 6×10^9 live AS-30D cells were incubated with 3 mg RH1-βG for 1 hr on ice, washed twice with PBS and separated into 3 fractions of 2×10^9 cells. One group of rats was i.p. injected with 2×10^8 RH1-βG-coated cells and then immediately i.p. injected with 3 hourly i.p. injections of BHAMG (7.5 mg/kg). BHAMG was added to a final concentration of 10 or 200 μM to the other 2 fractions of RH1-βG-coated AS-30D cells (each 2×10^9 in 30 mL complete medium) for 2 hr at 37°C. The cells were then washed twice with PBS, and 2×10^8 cells were i.p. injected into 2 groups of rats. Another group of rats was i.p. injected with 2×10^8 AS-30D cells that had been exposed *ex vivo* to 10 μM pHAM for 2 hr at 37°C. A final group of naive rats was not treated. All rats were challenged with 10^7 live AS-30D on days 50 and 51.

In vitro *cell depletion*

Naive or ADEPT-cured rats were i.p. boosted with 2×10^7 lethally irradiated AS-30D cells after 40–100 days. Peritoneal exudate cells (PEC) were collected 6 days later. RBCs were lysed in ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2). The cells were incubated in a 15 cm culture plate at 37°C for 1 hr to remove plastic-adherent cells. Ig^+ cells were removed by panning on a 15 cm culture plate coated with 100 μg/mL of goat anti-rat Ig. Nonadherent cells, referred to as enriched PEC, were employed in CTL assays. For depletion experiments, enriched PEC were incubated with medium alone or OX35, OX8 or 3.2.3 ascites (1:500) for 1 hr at 6–10–°C before the cells were washed 3 times with DMEM. Goat anti-mouse IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 15 min before the cells were washed once with medium. Depletion of labeled cells was carried out in BS separation columns according to the manufacturer's instructions (Miltenyi Biotec).

Flow cytometer analysis

T cells were identified by the rat CD5 T-cell marker,[14] whereas the MAb 3.2.3 (anti-CD16) was employed to identify NK cells.[15] $CD4^+$ and $CD8^+$ T cells were double-labeled as $CD5^+CD4^+$ and $CD5^+CD8^+$ cells,[16] respectively. Lymphocytes (5×10^5) in DMEM containing 0.5% bovine serum were incubated for 60 min at 4°C with first and second antibodies. Cells were washed and suspended in PBS containing 5 μg/mL propidium iodide before the surface immunofluorescence of 10^4 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA). Dead cells, identified by red propidium iodide fluorescence, were gated out. Fluorescence intensities were analyzed with Cell Quest Software (Becton Dickinson).

CTL assay

Cells (5×10^5 to 3×10^6 CHA, NMU, N1-S1 and C6) were labeled with 30 μCi/mL [^{3}H]-thymidine for 16 hr in complete medium. Labeled cells (5×10^3) were mixed with the indicated ratios of enriched PEC in 96-well round-bottom microtiter plates. Plates were centrifuged at 200*g* for 2 min and then incubated for 5 or 16 hr in a CO^2 incubator. Harvested cells were counted for radioactivity on a TopCount microplate scintillation counter (Packard Instrument Company, Meriden, CT). Specific killing was calculated by $100 \times (S - E)/S$, where S = cpm of cells in the absence of killers and E = cpm of cells in the presence of killers.[17]

In vivo *depletion of immune cells during ADEPT*

$CD4^+$ T cells were inactivated by i.p. injection of 1 mL OX38 and 1 mL OX35 ascites every day from day 0 to day 6 and every 2 days from day 8 to day 14. $CD8^+$ T cells were depleted by i.p. injection of 1 mL OX8 ascites on days 0, 2, 6 and 13. Both $CD4^+$ and $CD8^+$ T cells were depleted by injecting OX38, OX35 and OX8 ascites. NK cells were depleted by i.p. injection of 0.5 mL 3.2.3 ascites on days 0, 2, 6 and 13. Macrophages were inactivated by i.p. injection of 100 mg silica suspended in water on days 0, 6 and 13. Blood samples collected from rats starting on day 7 were characterized by immunofluorescence staining as described above. SD rats were i.p. injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 7, rats received i.p. injections of PBS or 300 μg RH1-βG followed 2, 3 and 4 hr later with 7.5 mg/kg BHAMG in PBS. The survival of animals was monitored for 100 days.

$CD4^+$ T-cell activity

SD rats were s.c injected with 100 μg OVA on days 1 and 11. Rats were then injected with 1 mL OX35 and OX38 ascites from days 22 to 28. Spleens were removed from control and depleted rats on day 29. RBCs were lysed in ACK buffer and enriched lymphocytes were obtained from spleen cells by removing plastic and Ig^+ adherent cells. Lymphocytes were enriched for T cells on a nylon wool column. Eluted cells (2×10^5) were mixed with 4×10^5 irradiated naive splenocytes (APC) and 10 μg/mL OVA in wells of a 96-well microtiter plate. After culture for 96 hr, 1 μCi/well of [^{3}H]-thymidine was added for 16 hr before the radioactivity of the wells was determined.

Macrophage activity

SD rats were s.c. injected with 100 μg βG in complete Freund's adjuvant on day 1 and boosted with 100 μg βG in incomplete adjuvant on day 15. Spleens were collected from the rats on day 34. T cells were isolated from splenocytes by treatment with ACK buffer and removal of plastic-adherent, Ig^+ and nylon wool adherent cells. Another group of rats was i.p. injected with 100 mg silica on days 24 and 31. Splenocytes or PEC (4×10^5) collected from silica-treated rats on day 34 were irradiated and then incubated for 96 hr with 10 μg/mL βG and 2×10^5 T cells. [^{3}H]-thymidine (1 μCi/well) was added for 16 hr before the radioactivity of the wells was measured in a TopCount scintillation counter.

NK cell activity

SD rats were i.p. injected with 0.5 mL 3.2.3 ascites on days 0, 2, 7 and 14. Both naive and depleted SD rats were i.v. injected with 500 μg polyinosinic-polycytidylic acid on days 7 and 14. RBCs in splenocytes that were collected 17 hr later were lysed with ACK

TABLE I – EFFICACY OF ADEPT THERAPY OF AS-30D ASCITES[1]

Experiment	Cures/total	
	Nontreated	ADEPT
1	0/6	19/20
2	0/6	15/16
3	0/4	12/12
4	0/4	11/11
5	0/6	21/22
6	0/6	24/24
7	0/4	7/8
8	0/4	15/16
9	0/6	30/30
10	0/2	2/2
Total	0/48	156/161

[1]SD rats were i.p. injected with 1.5×10^7 AS-30D cells on days 1 and 2. Treated rats were injected with 300 μg RH1-βG on day 7 followed 2, 3, and 4 hr later with 7.5 mg/kg BHAMG. Animals surviving for at least 60 days were considered cured.

buffer and then incubated with 5,000 ^{51}Cr-labeled Yac-1 cells. The radioactivity of 50 μL samples of culture supernatant collected after 6 hr was measured in a Topcount scintillation counter.

Statistical analysis

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

RESULTS

AS-30D hepatocellular carcinoma cells injected into syngeneic SD rats formed malignant ascites with 100% animal mortality within 3–4 weeks (Table I). ADEPT was performed 7 days after inoculation of AS-30D tumor cells by first i.p. injecting RH1-βG followed by injection of BHAMG, a glucuronide prodrug of aniline mustard. Table I shows that ADEPT cured 96.9% (156 of 161) of rats bearing established malignant ascites. The requirements for therapeutic efficacy by ADEPT were assessed in a separate experiment in which rats were treated 7 days after tumor inoculation. Table II shows that untreated rats had a mean survival time of 10.8 ± 0.63 days, showing that tumors were well advanced at the initiation of therapy.

Treatment of the advanced tumors with pHAM, the active product of BHAMG, produced 1 long-term survivor (>40 days) but did not significantly increase mean survival time. Effective treatment of advanced AS-30D tumors required combined treatment with RH1-βG immunoenzyme and BHAMG prodrug (ADEPT). Treatment with RH1-βG alone resulted in a slight but significant increase in mean survival time (15.5 ± 2.1 days, $p \leq 0.05$ compared with control rats) but did not produce any long-term survivors. Treatment with BHAMG alone did not provide antitumor activity. Combination treatment with RH1-βG and BHAMG (ADEPT), in contrast, produced 100% long-term survivors (mean survival times all > 40 days). Injection of the MAb RH1 did not produce significant antitumor activity regardless of whether BHAMG was also administered. Injection of a mixture of MAb RH1 and free βG (equivalent amounts as contained in the RH1-βG immunoenzyme) did not increase the mean survival times of the rats, whereas injection of RH1, βG and BHAMG produced 1 long-term survivor although mean survival time was not significantly increased. ADEPT significantly increased the mean survival times of tumor-bearing rats ($p \leq 0.05$) compared with all other regimens shown in Table II, clearly showing that effective treatment of advanced tumors depended on both the immunoenzyme and the prodrug components of ADEPT.

The development of protective immunity in rats cured by ADEPT was examined by i.p. injection of 2×10^7 viable AS-30D cells. Table III shows that cured rats were protected from a lethal challenge of AS-30D cells as soon as 15 days and for at least 100 days after termination of ADEPT. Immunization of up to 10^9 radiation-killed AS-30D cells failed to induce protective immunity to a subsequent challenge of live AS-30D cells (Fig. 1*a*), indicating that AS-30D cells were not highly immunogenic. Figure 1*b* shows that tumor growth in rats that were injected with immunoenzyme-coated AS-30D cells was not significantly delayed compared with rats that were injected with radiation-killed AS-30D cells after challenge with live AS-30D cells (24 ± 2.3 *vs.* 22 ± 2.8 days, $p = 0.3$). Thus, RH1-βG did not dramatically increase tumor cell immunogenicity.

TABLE II – SPECIFICITY OF ADEPT[1]

Group	First injection	Second injection	Mean survival (days ± SE)	Long-term survivors/total
Control	PBS	PBS	10.8 ± 0.63	0/4
Drug	PBS	pHAM	19.5 ± 8.5	1/4
Prodrug	PBS	BHAMG	11.8 ± 1.5	0/4
Immunoenzyme	RH1-βG	PBS	15.5 ± 2.1[2]	0/4
ADEPT	RH1-βG	BHAMG	>40[3,4]	4/4
Antibody	RH1	PBS	14.3 ± 3.3	0/4
Antibody + prodrug	RH1	BHAMG	14 ± 2.1	0/4
Antibody + enzyme	RH1, βG	PBS	10.8 ± 0.85	0/4
Antibody + enzyme + prodrug	RH1, βG	BHAMG	20.8 ± 8.2	1/4

[1]SD rats were i.p. injected with 1.5×10^7 AS-30D cells on days 1 and 2. On day 7, rats were injected with PBS or the indicated compounds followed 2, 3 and 4 hr later with PBS, prodrug (BHAMG) or drug (pHAM). Animals survival was followed for 40 days.–[2]Significantly greater than control group ($p \leq 0.05$).–[3]Significantly greater than all other groups ($p \leq 0.05$).–[4]Significantly greater than control group ($p \leq 0.0005$).

TABLE III – PROTECTIVE IMMUNITY INDUCED BY ADEPT[1]

Days after ADEPT therapy	Cures/total	
	Naive	ADEPT
15	0/4	4/4
30	0/4	4/4
45	0/4	4/4
100	0/8	8/8

[1]Naive rats or rats cured of AS-30D tumors by ADEPT were challenged with 2×10^7 live AS-30D cells at the indicated times. Rats surviving the tumor challenge for at least 60 days were considered cured.

Table IV shows that the development of antitumor immunity required administration of RH1-βG and BHAMG to rats that had established ascites tumors. AS-30D cells treated with RH1-βG followed by either a low dose (10 μM) or high dose (200 μM) of BHAMG before implantation into SD rats failed to protect animals from a lethal challenge of live AS-30D cells given 50 days later. Likewise, immunization of rats with tumor cells that were treated *ex vivo* with pHAM, the active metabolite of BHAMG, did not protect rats from a later tumor challenge. AS-30D cells that were saturated with RH1-βG *ex vivo* and implanted into the peritoneal cavity of rats before BHAMG was administered also failed to protect animals from a lethal challenge of AS-30D cells.

In vitro *analysis of effector cells*

Naive and ADEPT-cured rats were stimulated with an i.p. injection of radiation-killed AS-30D tumor cells, and the lytic activity of peritoneal lymphocytes isolated 6 days later was assayed. Lymphocytes isolated from ADEPT-cured but not from naive rats killed AS-30D target cells in a dose-dependent manner (Fig. 2). Killing was specific for AS-30D cells because lympho-

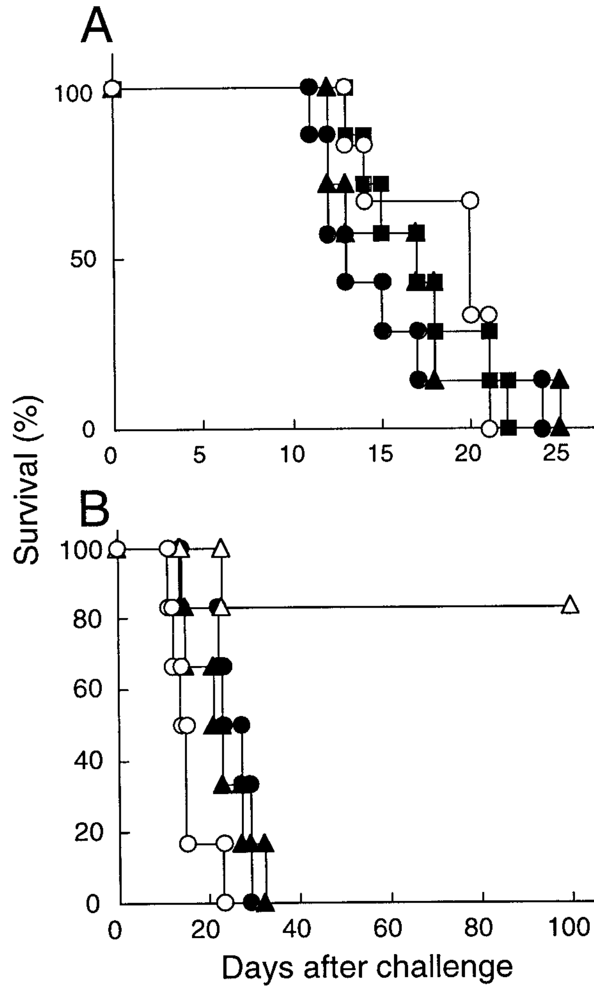

FIGURE 1 – Lack of protective immunity after immunization with irradiated AS-30D cells. (*a*) Groups of 6–8 SD naive rats were untreated (open circles) or i.p. injected with 10^7 (triangles), 10^8 (solid circles) or 10^9 (squares) radiation-killed AS-30D cells 100 days before challenge with live AS-30D cells. (*b*) Groups of 6 SD rats were untreated (open circles), injected with AS-30D tumors and treated by ADEPT (open triangles) or i.p. injected with 10^8 radiation-killed AS-30D cells without (solid triangles) or with prior exposure to RH1-βG (solid circles). Rats were challenged with live AS-30D cells 50 days later. Results show the survival of rats starting from the first day after AS-30D challenge.

TABLE IV – GENERATION OF PROTECTIVE IMMUNITY REQUIRES ADEPT TREATMENT OF ESTABLISHED TUMORS[1]

Treatment	Mean survival (days) ± SE	Long-term survivors[2]/total
ADEPT[3]	>55.9 ± 4.1[4]	7/8
Naive	23.0 ± 1.0	0/8
Ex vivo RH1-βG[5] *Ex vivo* 200 μM BHAMG[6]	27.3 ± 2.6	0/8
Ex vivo RH1-βG[5] *Ex vivo* 10 μM BHAMG[6]	29.6 ± 4.6	1/8
Ex vivo RH1-βG[5] *In vivo* BHAMG[7]	25.0 ± 0.8	0/8
Ex vivo 10 μM pHAM[6]	25.1 ± 2.0	0/7

[1]SD rats that received various treatments were challenged 50 days later with live AS-30D cells. Results show the survival of animals starting from the first day after AS-30D challenge.–[2]>60 days.–[3]SD rats were i.p. injected on days 1 and 2 with 1.5 × 10^7 viable AS-30D cells. Rats were treated on day 7 by i.p. injections of RH1-βG and BHAMG.–[4]Significantly greater ($p \leq 0.0005$) than naive rats.–[5]AS-30D cells were incubated with RH1-βG *ex vivo*.–[6]AS-30D cells were incubated with the indicated concentration of BHAMG or pHAM for 2 hr before the cells were i.p. injected into naive rats.–[7]AS-30D cells treated with RH1-βG were i.p. injected into rats followed by i.p. injections of BHAMG as in ADEPT.

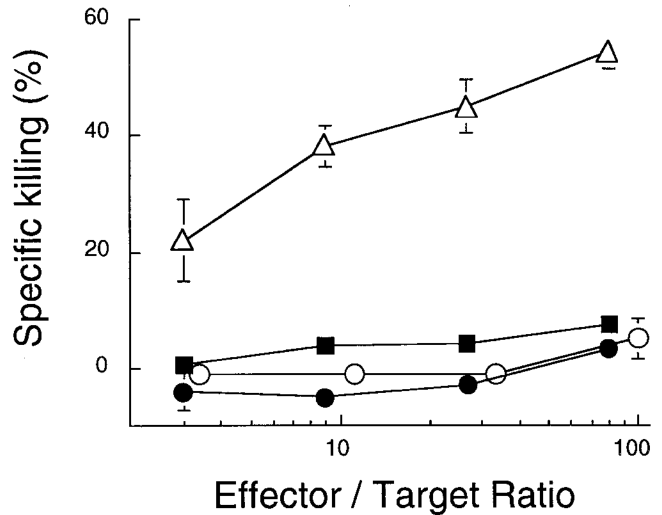

FIGURE 2 – CTL activity induced by ADEPT. Naive (open circles) or ADEPT-cured SD rats were i.p. boosted with radiation-killed AS-30D cells. Peritoneal cells isolated 6 days later were depleted of plastic-adherent and Ig^+ cells before they were assayed for cytotoxicity against AS-30D (triangles), NMU (squares) or C6 (solid circles) cells in a 16 hr CTL assay. Results represent mean specific killing values of quadruplicate determinations at the indicated effector to target ratios. Bars, SE.

cytes isolated from hosts cured of AS-30D ascites by ADEPT did not kill syngeneic NMU mammary adenocarcinoma or C6 glioma cells.

In vitro depletion was employed to delineate which subset of lymphocytes was responsible for killing AS-30D tumor cells. Naive and ADEPT-cured rats were stimulated by an i.p. injection of radiation-killed AS-30D cells, and PEC were isolated 6 days later. Large numbers of plastic-adherent cells were present in the peritoneal exudates; 50% and 88% of PEC from ADEPT-cured and naive rats bound to plastic, respectively. The remaining PEC was characterized by immunofluorescence staining. Figure 3*a* shows that 36% of the peritoneal lymphocytes isolated from naive rats were $CD8^+$ T cells and 60% were $CD4^+$ T cells. ADEPT-cured rats had fewer $CD4^+$ T cells (Fig. 3*c*) but far more NK cells (17.2%; Fig. 3*d*) compared with naive rats (1.8%) (Fig. 3*b*). Depletion of lymphocyte subsets with antibody-coated magnetic beads resulted in the removal of 99.5% of $CD4^+$ T cells (Fig. 3*e*), 98.1% of $CD8^+$ T cells (Fig. 3*g*) and 99.4% of NK cells (Fig. 3*f*).

The cytotoxic activity of antibody-depleted PEC from ADEPT-cured rats was examined against AS-30D tumor cells in both 5 hr (Fig. 4*a*) and 16 hr (Fig. 4*b*) assays. Depletion of $CD8^+$ T cells but not $CD4^+$ T cells or NK cells totally blocked (Fig. 4*a*) or reduced (Fig. 4*b*) killing of AS-30D target cells. Plastic-adherent and Ig^+ cells did not contribute to killing because there was no difference between the cytotoxicity produced by PEC before and after removal of these cells (Fig. 4*a*,*b*). PEC isolated from rats cured of AS-30D tumors by ADEPT also killed syngeneic N1-S1 hepatocellular carcinoma cells in a $CD8^+$ T-cell-dependent fashion (Fig. 4*c*).

In vivo *potentiation of antitumor immunity by ADEPT*

The relationship between antitumor immunity and ADEPT was investigated by *in vivo* depletion of specific populations of immune

cells. Injection of OX8 ascites completely depleted $CD8^+$ T cells (Fig. 5*b*), also shown by the disappearance of $CD5^+CD4^-$ T cells (Fig. 5*d*). Figure 5*f* shows that injection of OX35 and OX38 ascites modulated the expression of surface CD4 as previously demonstrated.[18] Figure 6*a* shows that treatment of rats with OX35 and OX38 functionally inactivated $CD4^+$ T cells because the proliferation of T cells from rats treated with OX35 and OX58 was

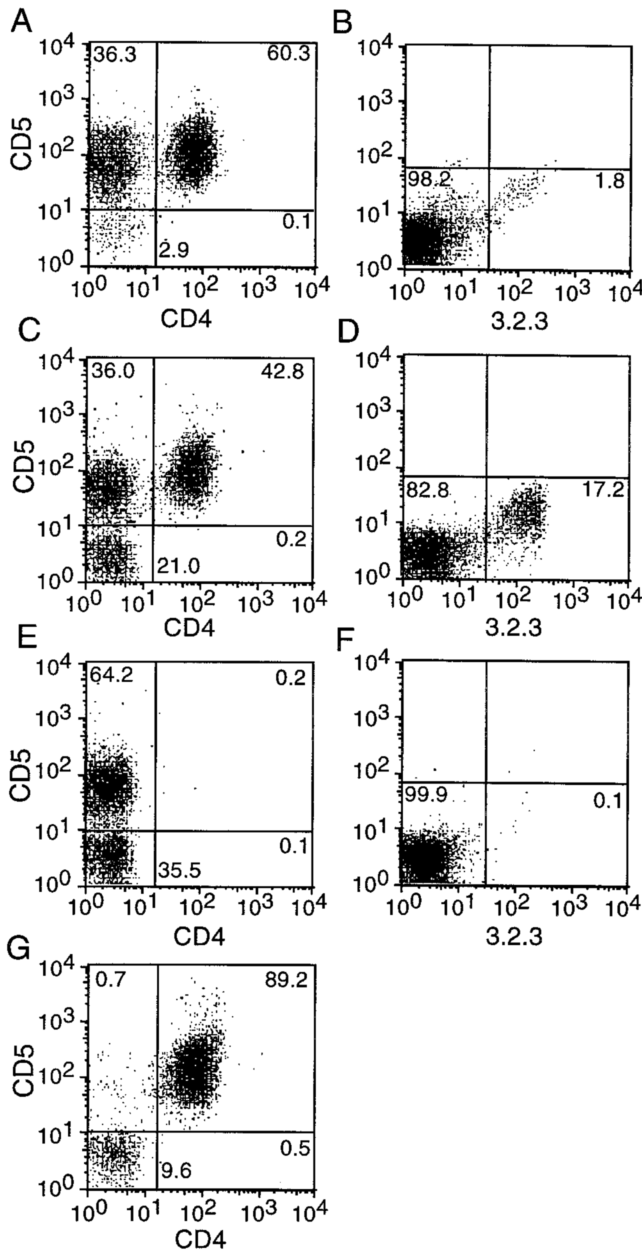

FIGURE 3 – *In vitro* depletion of lymphocyte subsets. Naive (*a*, *b*) or ADEPT-cured SD rats (*c–g*) were i.p. boosted with radiation-killed AS-30D cells. PEC isolated 6 days later were depleted of plastic-adherent and Ig^+ cells (*a–d*) as well as $CD4^+$ (*e*), $3.2.3^+$ (*f*) or $CD8^+$ (*g*) cells. Cells were stained with anti-CD5-PE and anti-CD4-FITC (first column) or MAb 3.2.3 and goat anti-mouse-FITC (second column) before the fluorescence of cells was determined in a FACScaliber flow cytometer.

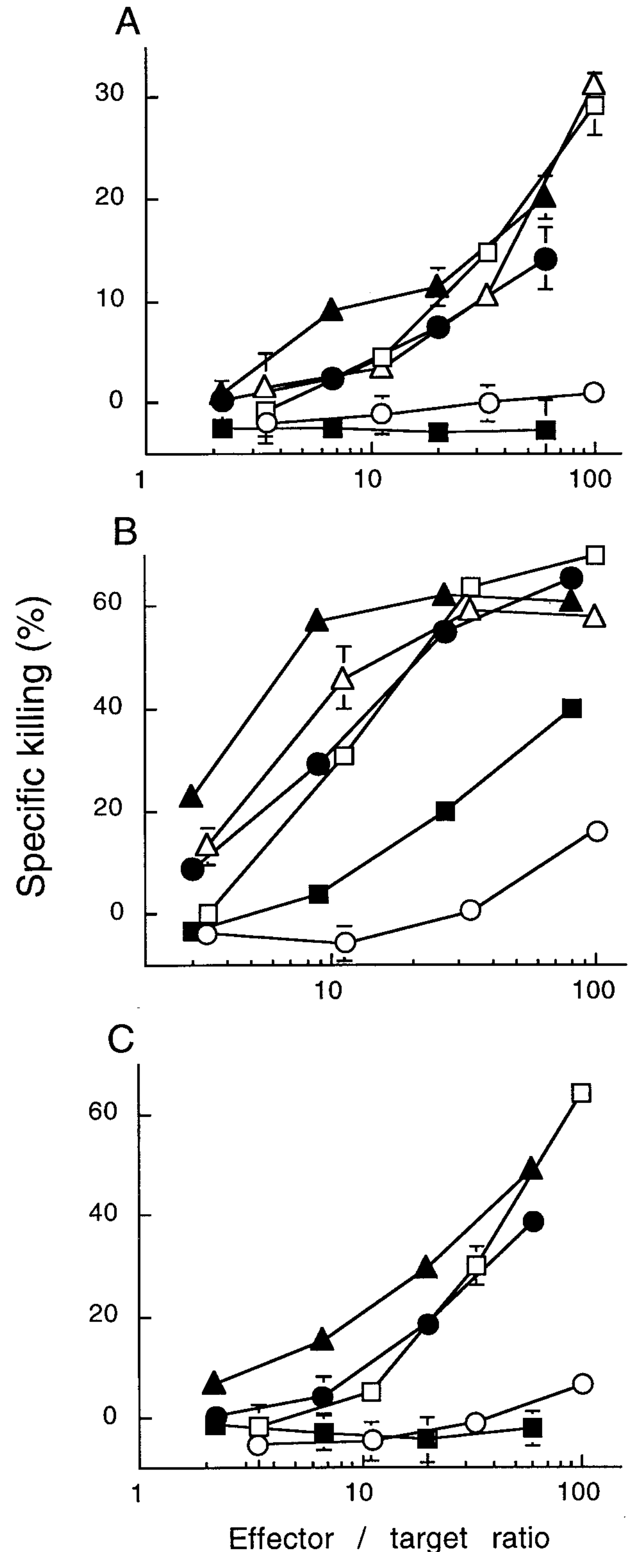

FIGURE 4 – Depletion of $CD8^+$ T cells decreases CTL activity against hepatocellular carcinoma cells. Naive (open circles) or ADEPT-cured rats were i.p. boosted with radiation-killed AS-30D cells, and PEC were isolated 6 days later. PEC were immediately assayed for CTL activity (open squares) or first depleted of plastic-adherent and Ig^+ cells (open triangles) as well as $CD4^+$ (solid triangles), $CD8^+$ (solid squares) or NK (solid circles) cells before they were assayed for CTL activity. (*a*) 5 hr assay, AS-30D cells as targets. (*b*) 16 hr assay, AS-30D cells as targets. (*c*) 5 hr assay, N1-S1 cells as targets. Results represent mean specific killing values of quadruplicate determinations at the indicated effector to target ratios. Bars, SE.

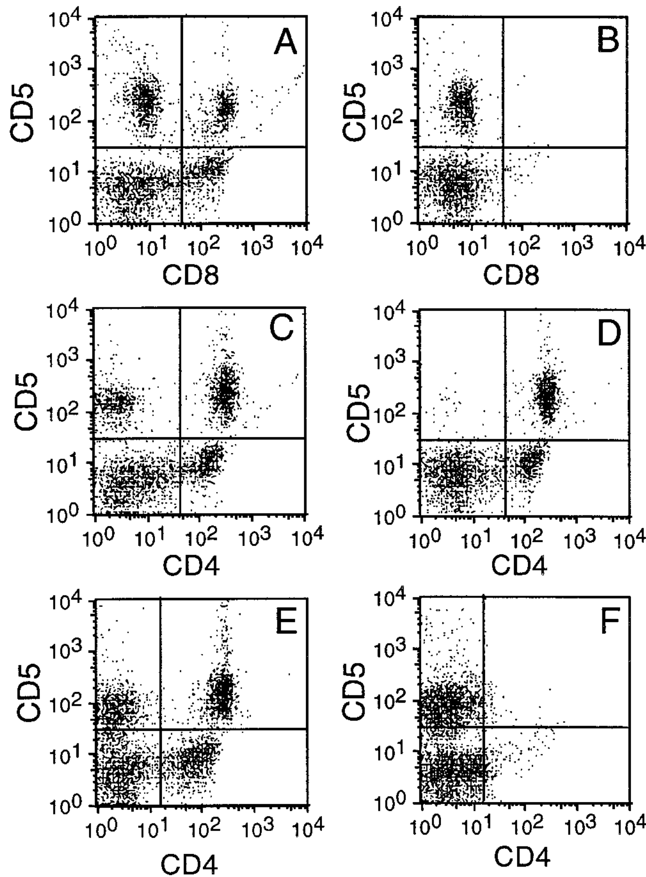

FIGURE 5 – FACs analysis of immune cells after antibody depletion. Blood cells isolated from SD rats before (*a*,*c*,*e*) or 1 day after (*b*,*d*, *f*) injection of OX8 (*b*,*d*) or OX35 plus OX38 (*f*) ascites were double-labeled with anti-CD5-PE and anti-CD4-FITC or anti-CD8-FITC as indicated. Immunofluorescence was determined on a FACScaliber flow cytometer.

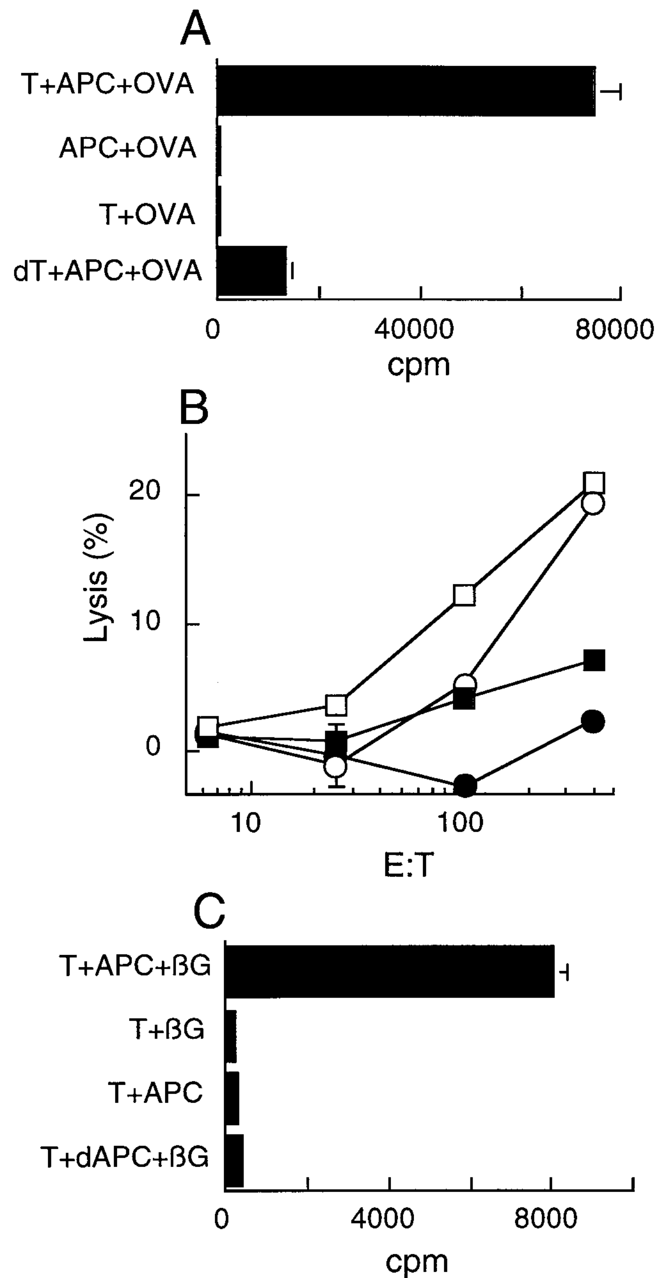

FIGURE 6 – Functional assay of effector cells after *in vivo* depletion. (*a*) $CD4^+$ T-cell proliferation. T cells isolated from the spleens of OVA-immunized rats (T+APC+OVA) or from OVA-immunized rats after 7 consecutive daily injections of OX35 and OX38 ascites (dT+APC+OVA) were incubated with irradiated antigen-presenting cells and ovalbumin for 96 hr before [^{3}H]-thymidine incorporation was measured. Assays performed without the addition of T cells (APC+OVA) or antigen-presenting cells (T+OVA) are indicated. (*b*) NK cell activity. SD rats were untreated (open symbols) or injected with 3.2.3 ascites on days 1, 2, 6 and 13 (solid symbols). Splenocytes collected from rats 17 hr after injection of polyinosinic-polycytidylic acid on days 6 (squares) and 13 (circles) were assayed for lysis of ^{51}Cr-labeled YAC-1 cells. (*c*) Macrophage activity. Splenocytes isolated from untreated (APC) or silica-treated (dAPC) rats were irradiated and mixed with β-glucuronidase (βG) and T cells isolated from β-glucuronidase-immunized rats (T). [^{3}H]-thymidine incorporation was measured 96 hr later.

reduced by 84% compared with untreated rats (Fig. 6*a*). Injection of 3.2.3 ascites reduced the activity of NK cells by 70–90% (Fig. 6*b*). The activity of macrophages was reduced by 98% in rats treated with silica (Fig. 6*c*). The antigen-presenting activity of PEC was also reduced by a similar amount after silica treatment of rats (results not shown).

Figure 7*a* shows the results of immune cell depletion during ADEPT. Untreated rats died from ascites within 25 days of tumor inoculation. Rats treated by ADEPT, in contrast, survived for 100 days and appeared to be free of disease. Depletion of NK cells or macrophages during ADEPT did not affect therapeutic efficacy because all rats were healthy after 100 days. Depletion of $CD4^+$ or $CD8^+$ T cells, in contrast, significantly ($p \leq 0.05$) decreased the efficacy of ADEPT with 50% of rats depleted of $CD4^+$ T cells and 75% of rats depleted of $CD8^+$ T cells dying within 100 days. Depletion of both $CD4^+$ and $CD8^+$ T cells totally abrogated the effectiveness of ADEPT ($p \leq 0.0005$) in this model with 100% of the rats dying by day 36. Rats that died after depletion of $CD4^+$ or $CD8^+$ T cells displayed disseminated tumors outside the peritoneal cavity and in the liver (Fig. 7*c*).

DISCUSSION

Antibody-directed enzyme prodrug therapy is attractive for cancer treatment because high concentrations of antineoplastic drug can be selectively generated at tumor cells to increase the therapeutic index of chemotherapy. Cure of disseminated disease, however, will probably require the generation of antitumor immunity. The role of immunity in ADEPT has remained unexplored because most studies have investigated human xenografts in immunodeficient mice.[7] We

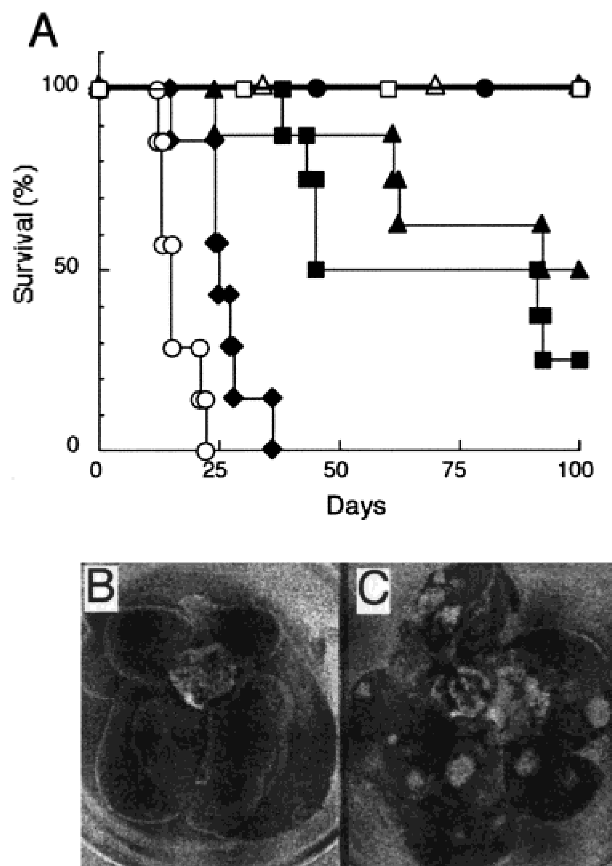

FIGURE 7 – *In vivo* depletion of cell subsets during ADEPT. (*a*) Groups of 7 or 8 rats bearing established AS-30D ascites were non-depleted (open squares) or depleted of NK cells (solid circles), macrophages (open triangles), $CD4^+$ T cells (solid triangles), $CD8^+$ T cells (solid squares) or both $CD4^+$ and $CD8^+$ T cells (diamonds) during ADEPT therapy. Control rats bearing AS-30D ascites (open circles) were not treated by ADEPT. Results show animal survival from the first day of tumor inoculation. Livers removed from rats after treatment by ADEPT without (*b*) or with depletion of $CD4^+$ T cells (*c*) are shown.

therefore employed a rat model of malignant ascites to investigate the role of antitumor immunity in ADEPT. The advantages of this model include the ability to propagate syngeneic tumors at an orthotopic site in immune competent animals, the effective treatment of advanced tumors by ADEPT and the ease of isolating tumor-infiltrating lymphocytes. Treatment of advanced tumors with drug (pHAM), prodrug (BHAMG), antibody (RH1), enzyme (βG) or immunoenzyme (RH1-βG) did not control tumor growth (Table II).

These results are in agreement with our previous findings.[12] Although this is a peritoneal tumor model, the combination of βG and BHAMG or a mixture of RH1, βG and BHAMG did not provide effective antitumor activity. We have previously shown that treatment of advanced malignant AS-30D ascites with a control immunoenzyme and BHAMG produced significantly more toxicity than treatment with RH1-βG and BHAMG.[12] Thus, efficacious treatment of advanced tumors requires combined treatment with both RH1-βG and BHAMG (Table II), showing that ADEPT provides selective antitumor activity.

Many of the prodrugs under development for ADEPT are alkylating agents[19] due to their high antitumor activity,[5] efficacy against tumor cells with defects in p53 and p21,[20] low likelihood of inducing multiple drug resistance[21] and short half-lives to limit diffusion of toxic drug from the tumor site.[22] Tumor treatment by ADEPT with alkylating agents, however, could prevent effective antitumor immunity due to dose-limiting leukopenia.[23,24] On the contrary, we found that rats cured by ADEPT with a prodrug of an alkylating agent developed potent antitumor immunity. More importantly, ADEPT synergized with the immune system to control metastatic disease.

Several lines of evidence indicate that antitumor immunity generated by ADEPT was not simply due to the intrinsic immunity of AS-30D cells employed in our study. Rats could not be protected from a lethal tumor challenge by immunization of large numbers of radiation- or drug-killed AS-30D cells. Immunoenzyme-coated tumor cells did not produce protection, showing that the tumor cells were not "haptenized."[25] In addition, AS-30D cells treated *ex vivo* with immunoenzyme and prodrug did not develop protective immunity. Protective immunity was also not observed after *in vivo* prodrug treatment of AS-30D cells that had been coated *ex vivo* with immunoenzyme. Our results indicate that antitumor immunity was only generated after progressively growing tumors were treated by ADEPT *in situ*. Further investigation is required to determine the relationship between the tumor microenvironment and ADEPT-generated immunity.

$CD8^+$ T cells were primarily responsible for killing tumor cells. $CD8^+$ T cells in rats cured of AS-30D tumors also lysed N1-S1 hepatocellular carcinoma cells but not mammary or glioma tumor cells, indicating that antitumor immunity was generated against a shared hepatocellular carcinoma antigen. Although large numbers of NK cells were found in the peritoneal cavity of ADEPT-cured rats (Fig. 3*d*), NK cells did not appear to play a role in the control of tumor growth because CTL activity was tumor specific, removal of NK cells did not decrease CTL activity against AS-30D cells and protective immunity was long lived. In addition, depletion of NK cells during ADEPT did not affect therapeutic efficacy. Lytic activity could not be completely blocked by removal of $CD8^+$ T cells in long-term (16 hr) CTL assays (Fig. 4*b*), suggesting that other cells such as neutrophils[26] or eosinophils[27] may have contributed to tumor cell killing.

An important finding of our study was that ADEPT did not cure tumor-bearing rats depleted of $CD4^+$ and $CD8^+$ T cells even though it was curative given 7 days after tumor transplantation in rats with an intact immune system. Depletion of either $CD4^+$ or $CD8^+$ T cells resulted in animal deaths due to disseminated tumor growth. Thus, either host immunity or ADEPT in the absence of an intact immune system did not control tumor growth whereas ADEPT cured immune competent animals, showing that ADEPT synergized with the immune system to provide greater antitumor efficacy. It should be noted that ADEPT with RH1-βG and BHAMG cured AS-30D solid tumors in immunodeficient mice,[9] showing that host immunity is not strictly required for the antitumor activity of ADEPT with BHAMG.

Enhanced antitumor immunity has been recently reported after suicide gene therapy of cancer employing herpes simplex virus-thymidine kinase (tk) to activate ganciclovir (GCV) or cytosine deaminase (CD) to activate 5-fluorocytosine (5-FC). Similar to our results, specific antitumor immunity beyond that produced by immunization with radiation- or drug-treated tumor cells has been demonstrated for some tumors treated by tk/GCV[28–31] or CD/5-FC.[32] In addition, T cells have been found to be involved in the induction or effector phases of the immune response in some cases.[31,32] The relevance to our results of the finding that GCV induction of tumor cell necrosis rather than apoptosis was related to upregulation of heat shock protein 70 expression and increased tumor cell immunogenicity[33] remains to be determined.

Several differences in the tumor immunity produced by ADEPT in our study and the immunity induced by prodrug activation by tk or CD are also apparent. Although both CD and βG are derived from *E. coli*, it appears that genetic modification of tumor cells with CD induces much stronger cellular immunity against the enzyme than does RH1-βG. For example, tumor cells transduced with CD can be rejected without prodrug treatment,[34] and immu-

nization of cells that express CD can protect against a challenge of unrelated tumor cells if they also express CD.[35]

We found, in contrast, that treatment of advanced tumors with βG or RH1-βG did not induce tumor rejection (Table II) and that *ex vivo* saturation of tumor cells with RH1-βG did not provide protective immunity even when combined with BHAMG treatment (Table IV). The high immunogenicity of CD is probably due to efficient presentation of CD peptides by MHC class I on tumor cells since CD is expressed in the cytosol of transduced tumor cells. RH1-βG, however, is targeted to an antigen on the surface of AS-30D tumor cells that is poorly internalized[36] and may not be effectively presented by MHC class I molecules although this has not been experimentally determined. Activation of 5-FC by CD has also been found to induce immunity mediated primarily by NK cells in some tumor models[37,38] whereas we did not find any evidence for the involvement of NK cells in the antitumor immunity generated by ADEPT.

Although it is difficult to make comparisons among different models, ADEPT with BHAMG may also produce more potent antitumor immunity compared with CD- or tk-mediated prodrug activation. Treatment of tk- or CD-transduced tumors with GCV or 5-FC in many instances produced similar antitumor immunity as immunization with radiation- or drug-treated tumor cells,[39,40] only weak or no CTL activity after prodrug treatment of tumors[37,41,42] and incomplete[29,39] or no[43,44] protective immunity. ADEPT, in contrast, generated long-lasting systemic antitumor immunity that could not be generated by immunization of radiation-treated tumor cells even with addition of immunoenzyme (Fig. 1), and strong $CD8^+$ T cell CTL activity was produced even without *in vitro* stimulation of T cells (Fig. 2). Further studies are required to determine the generality of these findings.

ADEPT may possess advantages compared with suicide gene therapy with GCV or 5-FC. Systemic delivery of transgenes to tumor cells remains technically challenging. Gene therapy of tumors therefore usually requires direct injection of tumors with viral or nonviral delivery systems, limiting therapeutic utility to nondisseminated tumors. Antibodies, however, can target tumor cells after systemic administration and have clearly demonstrated clinical utility for imaging[45] and therapy[46,47] with 8 antibodies already approved for clinical use.[48] ADEPT may also provide enhanced bystander killing of tumor cells since prodrug is activated extracellularly,[4,36] eliminating the need for active drug to diffuse to neighboring cells through gap junctions.[49]

Combination of cytokine genes with tk or CD transgenes has been shown to potentiate the antitumor immunity generated by suicide gene therapy in several models. For example, GCV treatment of tumors transduced with IL-2 and tk genes produced protective immunity whereas treatment of tk-transduced tumors did not.[43] NK and CTL activities were enhanced by 5-FC treatment of tumor cells that were transduced with both IL-2 and CD adenoviruses.[42] Similarly, a combination of GM-CSF adenovirus with CD transduction also increased CTL activity,[50] and generation of systemic immunity after GCV treatment required expression of both IFN-α and tk in another model.[44] These results suggest that stimulation of the immune system by cytokines or immunocytokines[51] may further increase the therapeutic potential of ADEPT. Our results also suggest that nonimmunogenic immunoenzymes should be employed to alleviate the need to administer immunosuppressive drugs to patients during ADEPT if repeated treatments are given.[52] Thus, immunoenzymes constructed with human β-glucuronidase[53–55] or human carboxypeptidase A1[56] may be preferable for the clinical application of ADEPT.

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