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Adriamycin(hydrazone)-antibody conjugates require internalization and intracellular acid hydrolysis for antitumor activity

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Summary. Adriamycin hydrazone (ADM-Hzn) immunoconjugates have previously been shown to exhibit antibody-directed antitumor activity in vitro and in vivo. In this report, the biological and biochemical properties of the mAb and linker were investigated. Conjugates prepared with two antibodies 5E9 [anti-(transferrin receptor)] and G28.1 (anti-CD37), (which internalize from the surface of target cells following binding) were more cytotoxic in vitro and had greater antitumor activity against Daudi B lymphoma tumor xenografts than a non-internalizing immunoconjugate prepared with mAb 2H7 (anti-CD20). In addition, the 13-acylhydrazone bond linking the drug to the mAb was labile at pH 5 and released unmodified ADM at a rapid rate ($t^1/_2$ = 2.5 h). Immunoconjugates prepared with an oxime linkage at the C-13 position were stable to acid and were not cytotoxic. These findings suggest that internalization of ADM-Hzn immunoconjugates and release of free ADM from the mAb in acidic intracellular compartments were important steps in the mechanism of action of ADM-Hzn immunoconjugates.

Key words: Monoclonal antibodies – Immunoconjugates – Adriamycin

Introduction

To be effective, a cytotoxic molecule must be transported from the extracellular space across the plasma membrane and into the nucleus or cytoplasm of the target cell. For a drug to be clinically useful, it must therefore take advantage of biochemical or uptake differences between the neoplastic and normal cell populations. Yet few exploitable differences have been found. The current interest in mAb-based serotherapies for the treatment of malignant disease is based on their potential to discriminate neoplastic cell populations from normal tissues. Major therapeutic approaches using mAbs are (a) the use of unconjugated mAb to activate or enhance endogenous defense mechanisms following antigen binding [13, 18], and (b) their use as carriers of radioisotopes [17, 21], toxins [19, 22–24], or chemotherapeutic drugs [7, 9, 11, 12, 32].

mAbs that bind to cell-surface receptors can enter the cell by a process similar to receptor-mediated endocytosis [10, 25, 26]. mAbs can also be internalized during membrane utilization or membrane biosynthesis [27]. Presumably, mAb-drug immunoconjugates would enter the cell via this same type of uptake mechanism. Receptor-mediated internalization of immunotoxins has been shown to be a necessary requirement for mAb-directed cytotoxicity [6, 20, 22, 29]. Antitumor activity may be associated with the ability of the mAb to substitute for the B (binding) chain of the toxin, and mimic B chain function for both binding and intracellular delivery of the A (toxic) chain. Those parameters that influenced internalization, such as the ability of the antibody to cross-link the receptor [24], the rate of intracellular delivery [23], antigen density [23], and the amount of surface antibody internalized [20], all affected cytotoxic efficiency.

The requirement for drug-antibody conjugates to be internalized for biological activity remains unclear. Evidence has been reported that methotrexate-mAb conjugates were processes in lysosomes [8, 9], and it has been suggested that delivery of *cis*-acontityl-linked anthracycline into acidic intracellular compartments via endocytic processes [7, 32] mediated cell killing. In contrast, mAb-*Vinca*-alkaloid conjugates may not require internalization for biological activity [26]. Recently our laboratories have described the synthesis of an Adriamycin (ADM) immunoconjugate in which the drug was attached to the antibody via an acid-sensitive 13-acylhydrazone (Hzn) linker [12]. Conjugates retained mAb-binding activity following drug conjugation and showed antibody-dependent (immunospecific) cytotoxicity in vitro [1, 12]. More important, when tested in human tumor xenograft models, ADM-Hzn conjugates demonstrated greater inhibition of tumor growth, increased potency and less systemic toxicity than

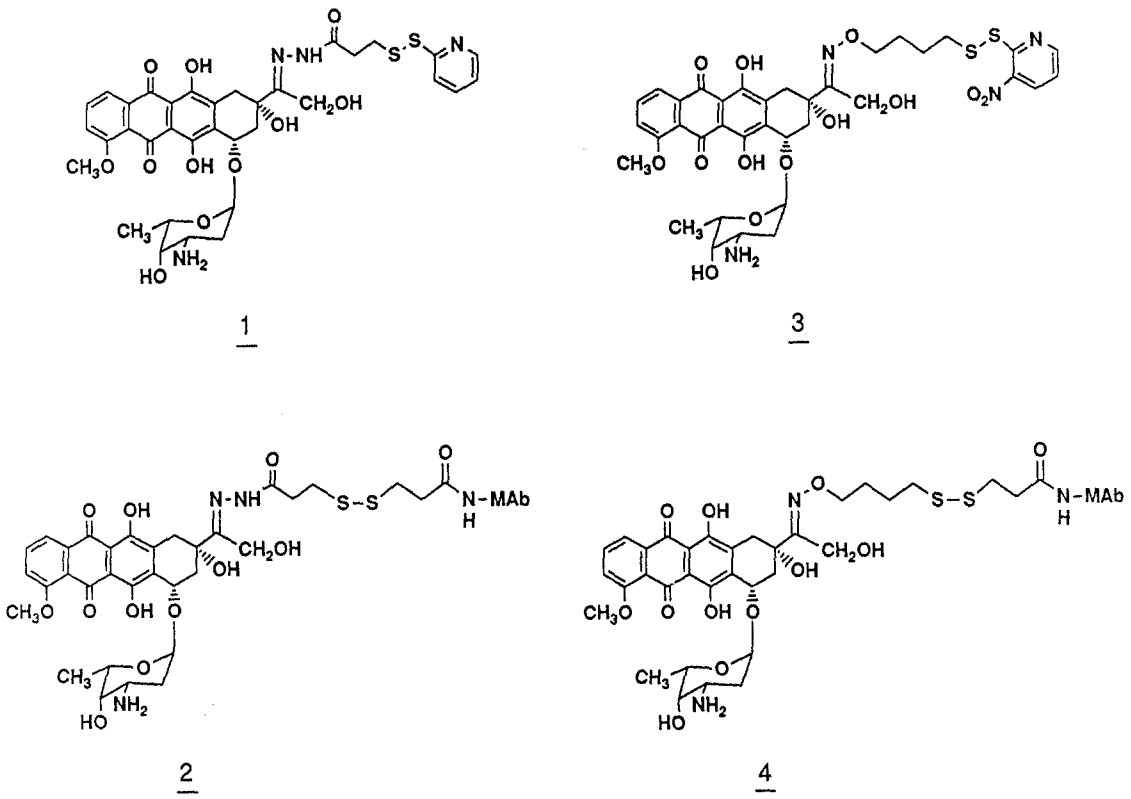

Fig. 1. Chemical structure of Adriamycin hydrazone (ADM-Hzn) (*1*) and its immunoconjugate (*2*). Chemical structure of ADM oxime (*3*) and its immunoconjugate (*4*)

The method of synthesising ADM-Hzn conjugates (condensation of ADM-Hzn with thiolated mAb) leads to the formation of a disulfide bond in the middle of the linker arm connecting mAb to drug. Reduction of the conjugate resulted in release from the protein of a 13-acylhydrazone thiol derivative of ADM while acid hydrolysis led to release of unmodified ADM [12]. The importance of either of these reactions for cytolytic activity has not been fully investigated. We speculated that internalization of conjugates into acidic intracellular compartments resulted in hydrolysis and intracellular release of active drug from the mAb protein. However, it has been suggested that unconjugated ADM does not necessarily have to enter the cell to have cytolytic activity [28], which may make it possible for anthracycline immunoconjugates to mediate activity through membrane binding only.

In the present study, we examined the internalization of ADM-Hzn immunoconjugates prepared with different mAbs. Conjugates that bound to cell-surface antigens that were internalized exhibited better antitumor activity in vitro and in vivo than non-internalizing conjugates. In addition, evidence is presented that shows that acid hydrolysis of the 13-acylhydrazone bond was also required for cytolytic activity. The proposed mechanism of action of killing mediated by internalization of mAb-ADM-Hzn conjugates is discussed.

Materials and methods

carcinoma), from I. Hellstrom, Oncogen] were produced and purified from tissue-culture supernatants by InVitron Corp. (St. Louis, Mo.). mAb 5E9 [IgG1, anti-(human transferrin receptor), ATCC no. HB21], mab 3A1 (IgG, pan T-cell antigen, ATCC no. HB2) and mAb 2H7 (IgG1, anti-CD20 B cell antigen, J. Ledbetter) were purified from ascites from BALB/c mice (Charles River, Inc., Ma.).

Cell lines. Daudi and Raji Burkitt's lymphoma cell lines were obtained from the ATCC. Cells were maintained in culture in RPMI-1640 medium containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Epitope density on Daudi cells was previously determined by Scatchard analysis using radiolabeled antibodies [11]. The Daudi cell line bound 2.8×10^5 molecules/cell of mAb 2H7, and 1.2×10^5 molecules/cell of mAb 5E9. mAb L6 and mAb 3A1 did not bind to the Daudi or Raji cell lines.

Preparation of ADM-Hzn immunoconjugates. ADM-Hzn immunoconjugates were prepared by attaching ADM Hzn, a 13-acylhydrazone derivative of ADM, to N-succinimidyl 3-(pyridyldithio) propionate (SPDP)-thiolated mAbs. The derivative, which contains a pyridyl-protected disulfide attached to the C-13 position of ADM via a hydrazone linkage (Fig. 1), was prepared as described previously [12]. Briefly, mAbs were reacted with SPDP (Pierce Chemical), dialyzed against phospate-buffered saline (PBS), and reduced with dithiothreitol (Sigma) to generate free thiol groups. Thiolated mAbs were reacted overnight at 4° C with ADM-Hzn and centrifuged at 5000 *g* to remove any precipitate formed during the coupling procedure. To remove unbound drug, immunoconjugates were exhaustively dialyzed against PBS, and passed over SM2 Biobead columns (BioRad Div., Richmond, Calif.). Conjugates used in this study contained less than 5% free drug as determined by HPLC analysis, and retained more than 95% of the original binding activity [12].

Preparation of ADM-oxime immunoconjugates. An ADM derivative having an oxime linkage at the C-13 position of the ADM molecule was

HCl to form the ADM derivative 13-[4-(3-nitropyridin-2-yl)-dithio]butyloximino-Adriamycin-HCl (ADM-oxime, Fig. 1). Immunoconjugates were prepared by reacting ADM-oxime with SPDP-thiolated mAbs and purified as described above.

High-pressure liquid chromatography (HPLC). The release of ADM under acidic conditions from either the hydrazone or oxime derivatives, as well as from the immunoconjugates was monitored and quantified by HPLC analysis. The derivatives were incubated at a concentration of 2 μM in 0.1 M sodium acetate buffer, pH 5, at 37° C. At various times aliquots were removed and chromatographed on a Waters HPLC system (model 510 pumps and a 680 gradient controller) using a μ-Bondapak C-18 10-μm column (3.8 × 300 mm). The mobile phase for elution of ADM-Hzn consisted of 68% triethylammonium formate buffer (0.05 M, pH 2.8) and 32% acetonitrile at a flow rate of 1.2 ml/min. ADM-oxime was chromatographed using a linear gradient from 68% buffer/32% acetonitrile to 30% buffer/70% acetonitrile over 13 min, after a hold for 7 min, at the initial conditions (flow rate 1.4 ml/min). Column effluents were monitored with an ABI model 980 fluorescence detector (excitation wavelength at 240 nm and emission wavelength of 550 nm). Data acquisition and integration were performed using Turbochrome software (PE Nelson) running on an IBM PS/2 model 70 computer.

Colony-formation assay. Cytotoxicity was determined by inhibition of colony formation in soft agar as previously described [12]. Daudi cells (1×10^5 cells/ml) were incubated for 2 h at 37° C with immunoconjugate or drug diluted in culture medium. Cells were washed and suspended at 5×10^3 cells/ml in RPMI-1640 medium containing 15% fetal bovine serum and 0.3% agarose (Marine Colloid). Suspensions of 1 ml were overlayed onto 0.4% agarose in six-well microtiter plates. Samples were incubated for 7–10 days at 37° C at 5% CO_2. Colonies were visualized by staining for 48 h at room temperature with 0.5 ml, 1 mg/ml *p*-iodonitrotetrazolium violet (Sigma). Colonies were counted using an Optimax 40–10 image analyzer.

Tumor models. Daudi solid tumor xenografts were serially passaged in 4- to 6-week-old BALB/c nu/nu mice (Harlan-Sprague Dawley) using 2×10^7 tumor cells implanted s.c. Tumors showed linear growth rates between 200 mm³ and 4000 mm³. The median tumor volume doubling time during exponential growth was 6.9 ± 0.8 days. When tumor volumes reached 400–600 mm³, mice were randomized into treatment groups of eight to ten animals each. Tumor volumes (V) were calculated using the formula:

$$V = \frac{L \times W^2}{2}$$

[where L = length (mm), W = width (mm)].

Modulation experiments – fluorescence assay. Antigenic modulation induced by mAb or mAb-ADM conjugate was assayed by indirect immunofluorescence using the Daudi B cell lymphoma cell line. Target cells (1×10^6 cells/ml) used to determine mAb-induced modulation were pulsed-labeled with the appropriate mAb (40–100 μg/ml) for 1 h at 4° C. Non-modulated cells were incubated under identical conditions with a similar concentration of a non-binding mAb (e.g. mAb L6). Cells were washed free from all unbound mAb, resuspended in culture medium and, at various times after incubation at 37° C, stained for residual cell-surface antibody by indirect immunofluorescence as described below. In certain experiments (Fig. 3), target cells were continuously exposed to mAb for 16 h at 37° C before being stained for residual cell-surface antibody.

mAb bound to the target cell population was determined using indirect immunofluorescence and quantified on an Epics V model 753 Flow Cytometer (Coulter Corp.). Cells, at various times during modulation at 37° C, were quickly cooled to 4° C, washed in ice-cold RPMI medium containing 2% fetal calf serum and incubated with 1 : 40 diluted fluorescein-isothiocyanate-conjugated goat F(ab)′2 anti-(mouse IgG) (Boehringer) for 30 min at 4° C. Cells were washed and the amount of cell-surface fluorescence quantified by flow cytometry. Background

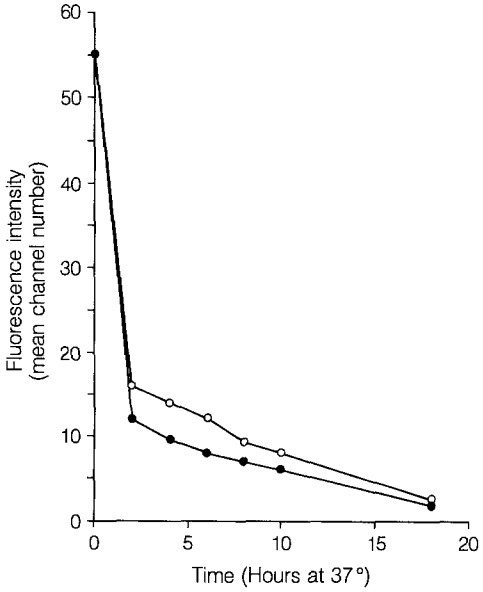

Fig. 2. Flow-cytometric analysis of internalization rates for mAb 5E9 (●) or 5E9-ADM-Hzn (○) conjugates. Cells were incubated with sufficient antibody protein (20 μg/ml) to saturate all available antigenic sites on Daudi cells. After 1 h incubation at 4° C, cells were removed from the cold, washed, and warmed to 37° C. At various times after warming, cells were removed and stained by indirect immunofluorescence for cell-surface antibody. Non-specific immunofluorescence was determined by incubation with non-binding mAb L6 before staining. Non-specific fluorescence (mean channel number) was subtracted from the mean channel number obtained using mAb 5E9 or conjugate

Modulation experiments-isotope assay. The amount of modulating antibody incorporated into the cell population during mAb modulation was determined using ^{125}I-labeled mAb. mAbs were radioiodinated with Na^{125}I (New England Nuclear, Ma.) using chloramine T. mAb-bound iodine was separated from free iodine by PD-10 column chromatography (Pharmacia Fine Chem.). Specific activities of the labeled preparations ranged from 2×10^5 to 8×10^5 cpm/μg protein.

Total antibody incorporated during modulation was determined by incubating Daudi cells (1×10^6 cells ml) with 40 μg/ml ^{125}I-labelled mAb (triplicates) in RPMI culture medium for 16 h at 37° C. Cell-bound radioactivity was separated from unbound radioactivity by overlaying the cell population onto 0.15 ml ice-cold 1 : 1 mixture of *n*-butyl phthalate (Fisher) and dinonyl phthalate (Kodak Chem.). Samples were then centrifuged in a Fisher model 235B microcentrifuge at 4° C for 1 min. Radioactivity in the pelleted cells was determined using a LKB model 1272 gamma counter. Non-specific binding was determined for each dilution by incubation with radioiodinated L6 antibody, and results (cpm) were subtracted from the values with the corresponding antibody.

Results

Modulation and internalization of mAb-ADM-Hzn conjugates

The ability of mAb or mAb-ADM-Hzn conjugates to induce antigenic modulation was demonstrated by staining for cell-surface antibody using indirect immunofluorescence. Daudi cells were incubated at 4° C with saturating concentrations of either mAb 5E9 or 5E9-ADM-Hzn conjugate. As shown in Fig. 2, warming the cells to 37° C

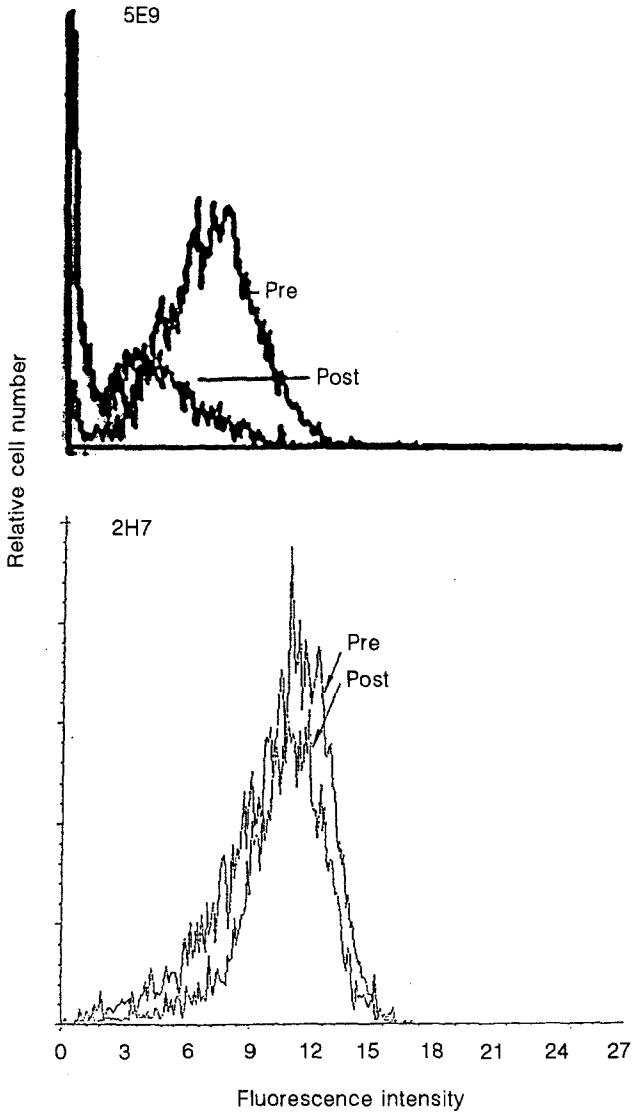

Fig. 3. Flow-cytometric analysis of Daudi lymphoma cells incubated with either mAb 5E9 (*upper panel*) or 2H7 (*lower panel*) before (premodulation) and after (post-modulation) incubation at 37° C for 16 h

Table 1. Binding of ^{125}I-labeled mAb to Daudi lymphoma cells before (premodulated) and after incubation at 37° C for 16 h (modulated)

mAb	Cell-bound radioactivity (cpm)	
	Premodulated[a]	Modulated[b]
5E9	9876	12174
2H7[c]	20569	24510
L6	841	745

[a] Cells were incubated with ^{125}I-labeled mAb (40 µg/ml) for 1 h at 4° C and washed free of unreacted mAb; cell-bound radioactivity was determined as described in Materials and methods

[b] Cells were continuously exposed to ^{125}I-labeled mAb for 16 h at 37° C; cell-bound radioactivity was determined as described in Materials and methods

[c] Results for mAb 2H7 were obtained separately from those for 5E9 and L6

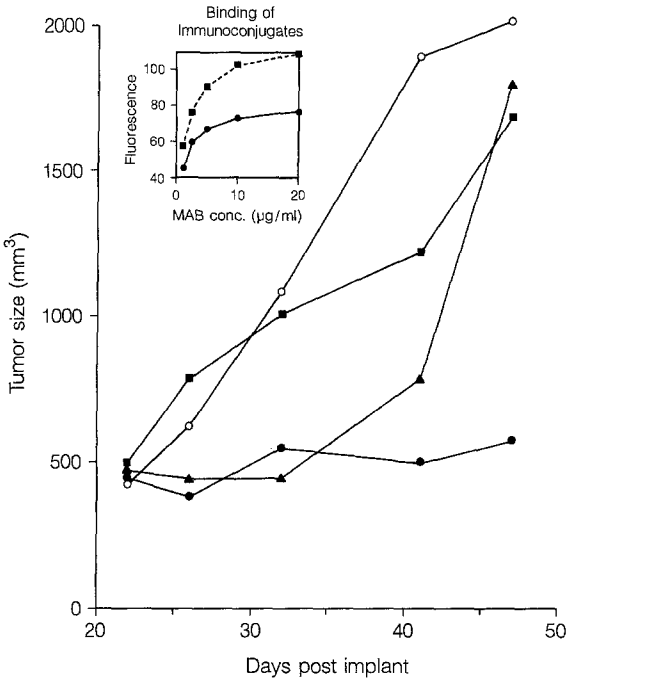

Fig. 4. Antitumor activity of Daudi tumor xenografts after inoculation of either internalizing, 5E9-ADM-Hzn or non-internalizing 2H7-ADM-Hzn, immunoconjugates. Mice (eight per group) were inoculated (i.p.) on days 21 and 26 after implant. 2H7-ADM conjugate (■) was inoculated at 600 mg/kg protein and 3.4 mg/kg drug. The 5E9 conjugate was inoculated at either a matching antibody dose (●): 600 mg/kg protein and 9 mg/kg drug, or at a matching drug dose (▲): 250 mg/kg protein and 4 mg/kg drug. Control mice (○) were untreated. *Inset*: antibody titration curves of the 2H7-ADM-Hzn (■) or 5E9-ADM-Hzn (●) conjugate on Daudi cells

surface was estimated to be approximately 2%–8%/min. In order to rule out the possibility that the loss of cell-surface antibody was not due to capping and subsequent sloughing of antibody from the cell surface, Daudi cells were incubated with saturating amounts of ^{125}I-labeled 5E9, and cell-bound radioactivity (Table 1) as well as cell-surface fluorescence (Fig. 3) was determined after incubation at 37° C. Essentially all of the radiolabeled antibody remained associated with cells, although cell-surface antibody could not be detected by immunofluorescence.

Similar results were obtained when saturating amounts of mAb 5E9-ADM-Hzn conjugates were used instead of mAb 5E9 (Fig. 2). The kinetics of antibody internalization was essentially the same for conjugated and unconjugated 5E9 mAb on the Daudi cell line. Not all mAbs were internalized following binding to a cell-surface antigen. This is illustrated in Fig. 3 and Table 1, where mAbs 2H7 and 5E9 (40 µg/ml) were incubated with Daudi cells and stained for cell-surface fluorescence before (premodulated) and after (post-modulated) incubation at 37° C for 16 h. In these experiments no loss of cell-surface antibody nor cell-bound radioactivity was observed when antigenic sites on the Daudi cell line were saturated using mAb 2H7. In contrast to mAb 2H7, mAb 5E9 showed a significant loss of cell-surface antibody (Fig. 3) without a comparable loss of cell-associated antibody (Table 1). These findings indi-

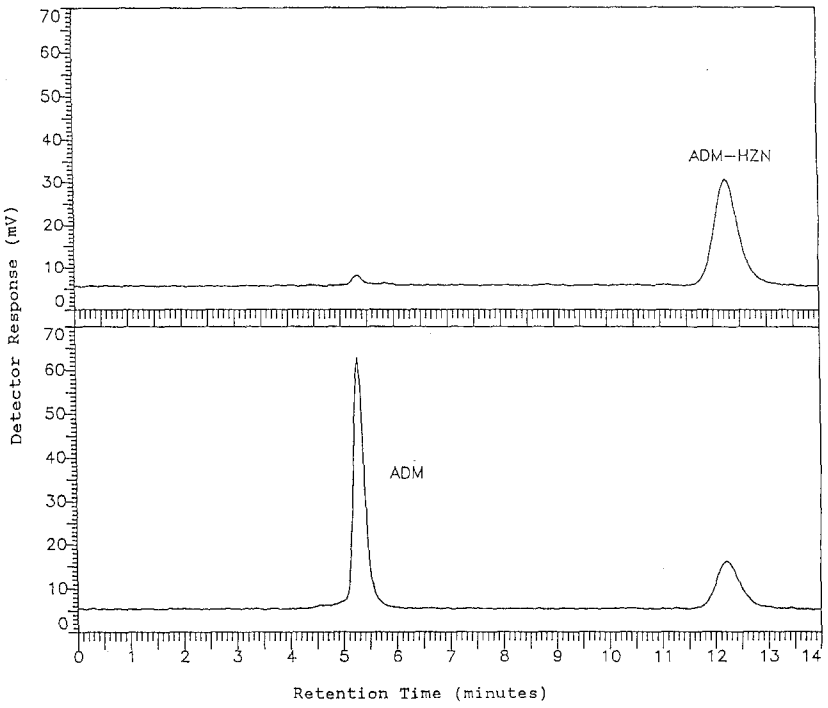

Fig. 5. Chromatogram of the ADM-Hzn before (*top*) and after (*bottom*) incubation for 4 h at 37° C, pH 5 sodium acetate buffer. Positions of ADM-Hzn and ADM are indicated on the chromatograms

Table 2. Cytotoxicity of Adriamycin hydrazone (ADM-Hzn) immunoconjugates in vitro[a]

Dose (μg/ml)	2H7-ADM-Hzn		5E9-ADM-Hzn		3A1 ADM-Hzn	
	Col./well[b]	I[c] (%)	Col./well	I (%)	Col./well	I (%)
6	64 ± 11	60	37 ± 8	75	84 ± 2	43
3	110 ± 2	25	85 ± 0.8	42	114 ± 13	22
1.5	149 ± 3.5	0	127 ± 13	14	149 ± 11	0
0.7	154 ± 27	–5.4	131 ± 8	11	155 ± 9	–6

[a] Immunoconjugates were incubated with Daudi cells for 1.6 h at 37° C prior to cloning in soft agar
[b] Colonies per well
[c] Inhibition (%) = [1–(treated/control)] × 100

body was internalized by the cell population, whereas the 2H7 antibody remained membrane-associated.

In vitro and in vivo antitumor activity

The necessity of internalization of ADM-Hzn immunoconjugates for cytotoxic or antitumor acitivity was investigated using conjugates prepared with either the internalizing 5E9 or non-internalizing 2H7 mAbs. The immunoconjugates bind to different antigens on the Daudi cell line, and there were approximately 2.5 times as many 2H7 binding sites as 5E9 binding sites (see insert, Fig. 4). In vitro cytotoxic activity was investigated by assaying the inhibition of colony formation. The 3A1-ADM-Hzn conjugate was used as a non-binding control. As shown in Table 2, cytotoxic activity of the 2H7 conjugate was comparable to that obtained with the non-binding 3A1 immences in colony inhibition were relatively small, this finding was consistent over the course of many experiments.

Fig. 4 compares antitumor activity of the non-internalizing (2H7) and internalizing (5E9) ADM-Hzn immunoconjugates on progressively growing Daudi tumor xenografts. Tumor growth inhibition mediated by the 5E9 conjugate was compared to that with the 2H7 conjugate at both matching drug (4 mg/kg) and at matching antibody (600 mg/kg) doses. Antitumor activity was significantly greater with the 5E9 conjugate at both conjugate doses, as compared to the non-modulating 2H7-ADM conjugate.

Hydrolysis of ADM-Hzn and ADM-oxime derivatives

mAb-ADM-Hzn conjugates were found to be stable at pH $\geq$7.4, but rapidly released ADM from the mAb at pH $\leq$6.5 [12]. As shown by the HPLC chromatogram in Fig. 5, ADM was the only product detected after acid hydrolysis of ADM-Hzn following a 4-h incubation at 37° C, pH 5. Comparison of the release of ADM from ADM-Hzn and ADM-oxime as a function of time at 37° C, pH 5, is shown in Fig. 6. The hydrazone derivative was rapidly converted to ADM ($t^1/_2$ = 2.5 h), while release of ADM from the ADM-oxime derivative was not detected at 6 h or even over a 24-h observation period. Immunoconjugates prepared from these derivatives demonstrated similar rates of hydrolysis (data not shown).

Cytotoxic activity of ADM-oxime-mAb conjugates

ADM-Hzn and ADM-oxime were linked to mAb G28.1, and both conjugates were shown to be internalized after

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