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DOXORUBICIN: MONOCLONAL ANTIBODY CONJUGATE FOR THERAPY OF HUMAN CERVICAL CARCINOMA

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Doxorubicin (Dox) was conjugated via a dextran linker to the F(ab')$_2$ fragment of monoclonal antibody (MAb) 1H10 which recognizes an antigen expressed on the surface of human cervical carcinoma cells and tissues. Drug-antibody conjugates (1H10-Dox) with a molar ratio of Dox to MAb ranging from 40:1 to 60:1 retained antigen-binding and pharmacological activities. Anti-tumor activity of the conjugate *in vitro* was evaluated by measuring inhibition of [5-^{3}H]-uridine incorporation into cellular RNA. 1H10-Dox was found to be 30 times more toxic to cervical tumor cells than a control MAb-Dox conjugate and 150 times more potent than Dox coupled to dextran. In addition, 1H10-Dox was less toxic to antigen-negative cells *in vitro*, suggesting that 1H10-Dox killing of cervical carcinoma cells was antibody-mediated. ^{125}I-labeled 1H10-Dox preferentially localized in solid human cervical carcinoma xenografts in athymic mice with tumor-to-blood ratios of 1H10-Dox reaching 17.9 after 24 hr and 32.8 after 48 hr. Treatment of athymic mice bearing human cervical tumors with 1H10-Dox resulted in a dose-dependent inhibition of tumor growth. Multiple administrations of 1H10-Dox at a dose corresponding to 20 μg doxorubicin significantly suppressed the growth of human cervical tumors in nude mice without significant side effects (weight loss), and this suppression was antibody specific. Both i.p. and i.v. administration of 1H10-Dox were found to be equally effective. Our results suggest that 1H10-Dox may be useful for the treatment of human cervical carcinoma.

Cervical carcinoma is a common lethal malignancy affecting women, especially African-Americans (Swanson *et al.,* 1983) and Asian women (Yeh, 1985). Cervical carcinoma is still a serious problem requiring continued attention, despite the widespread use of Papanicolaou smears (Murphy *et al.,* 1988) and a number of therapeutic trials (John, 1984). Moreover, most patients with advanced or metastatic lesions are not amenable to surgery or irradiation and can be treated only by systemic chemotherapy. Chemotherapy, however, is often prematurely discontinued because of drug toxicity and related side effects. Efficient targeting of chemotherapeutic drug to the cancerous area could be of great benefit for patients with advanced or metastatic cervical carcinoma.

The development of MAbs that preferentially recognize tumor cells has generated interest in targeting a variety of toxic materials to tumors (Ghose *et al.,* 1983; Vitetta *et al.,* 1983). The use of immunoconjugates for accurate delivery of lethal agents to human tumor cells may allow the creation of new treatment modalities with reduced side effects and decreased incidence of drug resistance. Several studies investigating the use of chemotherapeutic drugs attached to antibodies for cancer treatment are under active investigation (Durrant *et al.,* 1987; Smyth *et al.,* 1987; Tiandra *et al.,* 1989).

Doxorubicin, due to its potency against solid tumors, is a commonly used chemotherapeutic agent for the treatment of cervical carcinoma (Levi, 1984). Its major drawback, as with other cytotoxic agents, is that it is also toxic to normal proliferating cells. In recent years, there has been increasing interest in coupling doxorubicin or daunomycin to either polyclonal or monoclonal antibodies specific for tumors (Konno *et al.,* 1987; Dillman *et al.,* 1988; Yang *et al.,* 1988; Yu *et al.,* 1988), in order to maximize the concentration of drug delivered to the tumor site while minimizing toxicity to normal tissues.

In our study, doxorubicin was coupled to MAb 1H10 F(ab')$_2$ to form an immunodrug conjugate against human cervical tumors. MAb 1H10 is a murine IgG$_3$ antibody that reacts with several human cervical carcinoma cell lines and other carcinoma cell lines (Yu *et al.,* 1991). MAb 1H10 also reacts with more than 40% of cervical carcinoma tissues, but does not react with normal tissues tested, including normal cervix, liver, spleen, ovary, breast, colon, bladder, kidney, endometrium, cerebrum, esophagus, RBCs and lymphocytes (Roffler *et al.,* 1991*a*). An immunoconjugate formed between MAb 1H10 and *Pseudomonas* exotoxin has been shown to specifically kill cervical carcinoma cells *in vitro* and *in vivo* (Roffler *et al.,* 1991*b*). In this work, we have synthesized conjugates in which Dox was conjugated to oxidized dextran and then linked to MAb 1H10 F(ab')$_2$ to obtain a high level of drug loading on the antibody. We describe here the properties of 1H10-Dox and show that this conjugate can specifically kill human cervical tumor cells *in vitro.* We also show that 1H10-Dox preferentially localizes in human cervical tumor xenografts and can suppress the growth of xenografted tumors *in vivo.*

MATERIAL AND METHODS

Reagents

Doxorubicin was kindly supplied by Farmitalia, Milan, Italy. Dextran T-10 (molecular weight = 10 kDa), pepsin, fluorescein isothiocyanate, sodium periodate and sodium borohydride were purchased from Sigma, St. Louis, MO. Sephacryl S-300 gel was purchased from Pharmacia, Uppsala, Sweden. [5-^{3}H]-uridine (26 Ci/mmol) was purchased from Amersham Aylesbury, UK. Goat anti-mouse IgG F(ab')$_2$-specific horseradish peroxidase and goat anti-mouse IgG-FITC conjugates were purchased from Organon, Durham, NC.

Cell lines

All cells used for *in vitro* cytotoxicity and establishment of xenografted tumors were maintained in RPMI medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. CaSki human cervical carcinoma cell line was kindly provided by Dr. R.A. Pattillo, Medical College of Wisconsin, Milwaukee. H2669 human melanoma cell line was kindly provided by Dr. I. Hellström, University of Washington,

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Abbreviations: MAb, monoclonal antibody; Dox, doxorubicin; 1H10-Dox, conjugate of 1H10 antibody F(ab')$_2$ fragment with dextran-linked doxorubicin; 12.8-Dox, conjugate of 12.8 antibody F(ab')$_2$ fragment with dextran-linked doxorubicin; IC_{50}, concentration of test sample causing 50% inhibition of cellular RNA synthesis relative to control cell; Vt/Vc, ratio of tumor volumes in treated and control mice; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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Seattle, WA. COLO 205 human colon carcinoma cell line was obtained from ATCC, Rockville, MD.

Antibodies

MAb 1H10 (IgG_3) was developed in our laboratory using CaSki human cervical carcinoma cells as antigen source (Roffler *et al.,* 1991*a*). Mab 12.8, used as a control IgG_3 antibody, was also generated in our laboratory using COLO 205 human colon carcinoma cells as antigen source (Hwa, 1986). Antibodies were purified by protein A-Sepharose chromatography (Ey *et al.,* 1978) from ascites produced in BALB/c mice or from culture supernatant obtained from hybridomas grown in serum-free medium using an Opticell 5200R cell culture system (Charles River, Wilmington, MA). MAb 1H10 and 12.8 activities were determined by enzyme immunoassay using 96-well microtiter plates coated with CaSki or COLO 205 cells respectively. Plates were pre-treated with 50 μl/well of 10 μg/ml polylysine in PBS for 30 min before wells were coated with 10^5 CaSki or COLO 205 cells/well. Plates were centrifuged at 1500 *g* for 5 min and cells were then fixed with 0.5% gluteraldehyde in PBS for 15 min at room temperature. Wells were blocked with 0.1 M glycine, 0.01% BSA in PBS followed by 1% skim milk in PBS. Serial dilutions of antibody (50 μl/well) in 0.05% BSA/PBS were added to wells for 1 hr at 37°C. After washing wells 3 times with PBS containing 0.05% Tween 20, 50 μl/well peroxidase goat anti-mouse IgG $F(ab')_2$-specific conjugate (1:1500) was added for 1 hr at 37°C. Plates were washed 3 times with 0.05% Tween 20/PBS and twice with PBS, and bound peroxidase activity was measured by adding 100 μl/well ABTS solution (0.4 mg/ml 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, 100 mM phosphate-citrate, pH 4.0) for 15 min. Absorbance (405 nm) of wells was measured in a Molecular Devices (Menlo Park, CA) microplate reader.

Preparation of $F(ab')_2$ fragments

$F(ab')_2$ fragments of MAb 1H10 and 12.8 were prepared by pepsin digestion. After dialyzing purified antibody overnight against 0.1 M sodium acetate, pH 6.0, pH was adjusted to 4.0 with acetic acid and antibody was digested with a 1:100 (wt/wt) ratio of pepsin/IgG for 12 min at 37°C. Rapid digestion was necessary to prevent degradation of IgG_3. Pepsin was deactivated by raising the pH to 8.0 with 1.5 M Tris HCl and undigested IgG was removed by affinity chromatography on Sepharose CL-4B protein A, while free pepsin and small peptides were removed by size-exclusion chromatography on Sephacryl S-300 gel.

Conjugation of doxorubicin to MAb

Purified MAb 1H10 and 12.8 $F(ab')_2$ fragments were conjugated with doxorubicin by the dextran bridge method (Hurwitz *et al.,* 1975, 1979) with some modifications. Briefly, dextran T-10 was 25% oxidized to polyaldehyde-dextran with sodium periodate, extensively dialyzed against water, lyophilized, and stored at 4°C. Oxidized dextran was incubated with Dox overnight at room temperature. $F(ab')_2$ fragments (in 0.15 M sodium-bicarbonate buffer, pH 9.5) were then added to the mixture and the reaction was allowed to continue overnight at 4°C. The ratio of oxidized dextran, Dox, and immunoglobulin was 6:1:1 by weight. The resulting conjugates were reduced with a small excess of sodium borohydride. Antibody-doxorubicin conjugates were purified by Sephacryl S-300 gel filtration.

Characterization of conjugates

1H10-Dox composition was determined by protein analysis of antibody (Bradford, 1976) and spectrophotometric analysis (at 476 nm) of drug assuming an extinction coefficient $\epsilon^{1\%}_{1cm}$ of 173 (Yang *et al.,* 1988). Molecular weight of 1H10-Dox was estimated by SDS-PAGE under non-reducing conditions. Stability of 1H10-Dox and absence of free Dox were determined by gel filtration and thin-layer chromatography. TLC analysis was performed on aluminum sheets pre-coated with silica gel containing fluorescent indicator (Merck, Darmstadt, Germany) and developed with either 1-butanol/acetic acid/water (4/1/5) or methylene chloride/methanol/water (100/20/2). Quantitative tumor-binding activity of conjugates was measured by enzyme immunoassay as described above.

In vitro *cytotoxicity of 1H10-Dox*

The *in vitro* effect of 1H10-Dox was quantified by assaying inhibition of cellular RNA synthesis. Inhibition of [5-^{3}H]-uridine incorporation into CaSki cells (antigen-positive) or H2669 cells (antigen-negative) was measured as reported by Deguchi *et al.* (1987) with some modifications. Briefly, 2×10^4 cells/well were plated in 96-well microtiter plates overnight. The cells were then exposed to various concentrations of test substances for 16 hr at 37°C in a humidified 5% CO_2 incubator. After 16 hr, 1 μCi of [5-^{3}H]-uridine was added for 2 hr. Incorporation of [5-^{3}H]-uridine into cellular RNA was measured in a Beckman (Palo Alto, CA) LS 5801 scintillation counter after washing cells 3 times with 6% trichloroacetic acid. Results are expressed as percentage inhibition of [5-^{3}H]-uridine incorporation compared with untreated cells by the following formula:

$$\% \text{ inhibition} = \frac{\text{cpm of the control} - \text{cpm of the sample}}{\text{cpm of the control}} \times 100.$$

Internalization of MAb 1H10 and 1H10-Dox

The ability of MAb 1H10 to enter CaSki cells was examined by both indirect and direct immunofluorescence. In the indirect method, viable CaSki cells were washed twice with RPMI medium and re-suspended at 2×10^7 cells/ml in RPMI medium. Cells (100 μl) were incubated with 20 μl MAb 1H10

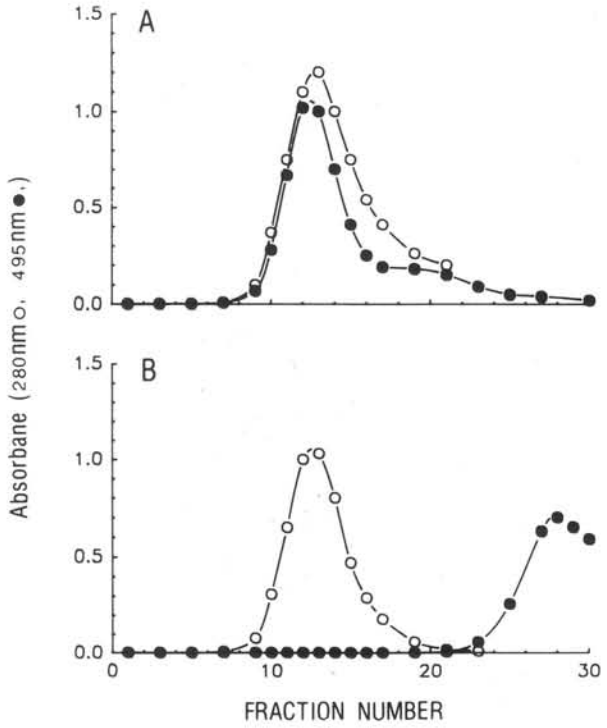

FIGURE 1 – Gel-filtration analysis of 1H10-Dox conjugate. *(a)* MAb 1H10 $F(ab')_2$ (absorbance at 280 nm, ○—○) and doxorubicin (absorbance at 495 nm, ●—●) linked via oxidized dextran eluted from a Sephacryl S-300 column as a single peak. *(b)* A mixture of MAb 1H10 $F(ab')_2$, doxorubicin and unoxidized dextran eluted from a Sephacryl S-300 column as separate peaks.

ascites for 1 hr at 4°C to saturate antigen sites on the cell membrane. CaSki cells incubated with non-binding antibody ascites served as a negative control. Cells were then washed once with cold RPMI medium, resuspended in 100 μl medium, and maintained at 4°C or incubated at 37°C for 30 min to allow internalization of surface antibody. Cells were then held at 4°C for 90 min to minimize differences in surface binding due to temperature (Johnstone *et al.,* 1990). Cells were washed once with PBS and resuspended in 200 μl PBS containing goat anti-mouse IgG-FITC conjugate (1:100) for 1 hr at 4°C. After being washed twice with cold PBS, cells were resuspended in PBS containing 100 μl/ml glycerol and 100 μg/ml o-phenylenediamine and observed under a fluorescent microscope.

The ability of FITC-labeled MAb 1H10 $F(ab')_2$ to enter CaSki cells was also examined. Mab 1H10 $F(ab')_2$ fragments were labeled with fluorescein isothiocyanate to a molar ratio of 3.6 FITC per $F(ab')_2$ (Goding, 1983). Viable CaSki cells were washed with RPMI medium and cooled to 4°C. FITC-labeled MAb 1H10 was added to 2×10^6 CaSki cells in 100 μl RPMI medium to a final concentration of 25 μg/ml for 1 hr at 4°C. Cells were then washed with cold medium and incubated at 4°C or 37°C for 30 min. After holding the cells at 4°C for an additional 90 min, cells were incubated twice with 100 μl MAb 1H10 ascites at 4°C to compete surface-bound FITC-labeled MAb 1H10 from the surface of cells. Cells were then processed as above for microscopic observation.

Internalization of 1H10-Dox was also investigated. 1H10-Dox (50 μl) was incubated with 1.5×10^6 viable CaSki cells in 150 μl RPMI medium for 2 hr at 4°C or 37°C. Cells were subsequently washed twice with cold PBS and surface 1H10-Dox was removed by incubating cells twice with excess MAb 1H10 for 15 min at 4°C. Cells were then processed as above and doxorubicin fluorescence was directly observed under a fluorescent microscope.

Biodistribution of 1H10-Dox

1H10-Dox was radiolabeled with ^{125}I, using the Chloramine T method, to a specific activity of about 0.06 μCi/μg. Iodinated conjugate was tested by direct binding to CaSki and H2669 cells to assure maintenance of immunoreactivity and specificity. BALB/c nude mice bearing established subcutaneous CaSki tumors were injected i.v. with 20 μg radiolabeled 1H10-Dox. Mice were killed at different times and blood samples and organs were removed, weighed and counted for radioactivity in glass tubes in a Packard (Meriden, CT) 5650 gamma counter for 1 min. Between 40 and 350 mg tissue was counted and the radioactivity of samples was over 1300 cpm in all cases.

In vivo *activity of 1H10-Dox*

In vivo efficacy of 1H10-Dox was determined in nude mice bearing subcutaneous solid human cervical tumors. Female BALB/c *nu/nu* mice weighing 20 to 25 g were injected s.c. in the right flank with 10^7 exponentially-growing CaSki cells. Treatment was initiated after allowing tumors to grow 5 days. Groups of 5 mice were randomly selected and treated i.p. or i.v. with 1H10-Dox or control substances. The volume for i.p. injections was 15 ml/kg body weight, that for i.v. injections was adjusted to 5 ml/kg body weight. The dosages and time-course of treatment are indicated in figure legends. Doses of 1H10-

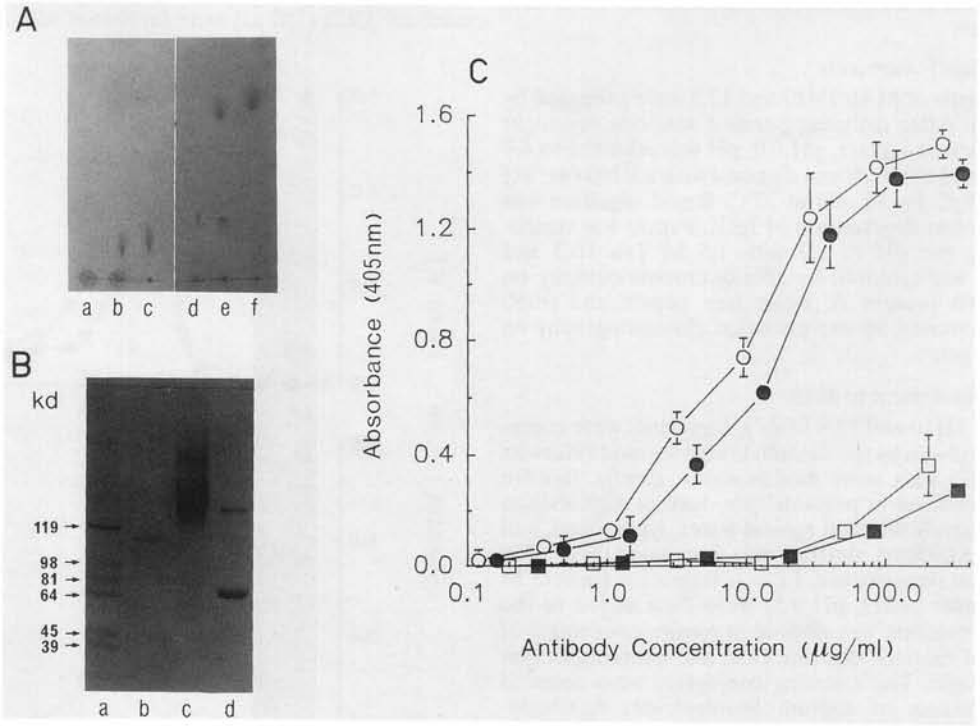

FIGURE 2 – Characterization of 1H10-Dox conjugate. *(a)* Thin-layer chromatography of 1H10-Dox. 1H10-Dox (lanes a, d), a mixture of 1H10-Dox and unconjugated Dox (lanes b, e) or free Dox (lanes c, f) were spotted on aluminium sheets pre-coated with silica gel and developed with methylene chloride/methanol/water (100/20/2) (lanes a, b, c) or 1-butanol/acetic acid/water (4/1/5) (lanes d, e, f). 1H10-Dox did not migrate in the methylene chloride/methanol/water system and migrated more slowly than Dox in the butanol/acetic acid/water solvent. *(b)* SDS-PAGE analysis of 1H10-Dox conjugate. Samples were electrophoresed on a non-reduced polyacrylamide gel and stained with Coomassie blue R-250. Lane a, pre-stained molecular weight standard; lane b, MAb 1H10 $F(ab')_2$; lane c, 1H10-Dox; lane d, MAb 1H10 IgG and BSA; kDa, molecular weight in thousands. *(c)* Enzyme immunoassay measurement of antigen-binding activities of MAb 1H10 $F(ab')_2$ (○), 1H10-Dox (●), MAb 12.8 $F(ab')_2$ (□) and 12.8-Dox (■) to CaSki cervical carcinoma cells. Means and standard errors of 3 determinations are shown.

Dox from different batches were adjusted to provide equivalent amounts of Dox in all experiments. Tumor size and body weight were measured twice a week. Tumor volumes were estimated by:

$$\text{tumor volume} = \frac{\text{height} \times \text{length} \times \text{width}}{2}.$$

Statistical analysis of results employed the Student's *t*-test.

RESULTS

Preparation and characterization of 1H10-Dox

Doxorubicin was conjugated to MAb 1H10 F(ab')$_2$ via a dextran bridge. Unconjugated Dox was removed from 1H10-Dox by gel filtration on Sephacryl S-300. 1H10-Dox eluted from the gel-filtration column as a symmetrical peak with drug absorbance at 495 nm superimposed upon antibody absorbance at 280 nm (Fig. 1*a*). A mixture of drug, monoclonal antibody and non-oxidized dextran eluted from the gel filtration column as separate peaks (Fig. 1*b*), indicating that Dox and antibody were not bound by simple physical association of drug and antibody. 1H10-Dox that was not reduced with sodium borohydride, in contrast, eluted from the gel filtration column as 2 peaks, indicating that reduction is necessary for immunoconjugate stability (data not shown). The stability and purity of 1H10-Dox was further verified by thin-layer chromatography. No free Dox was detected in 1H10-Dox preparations using 2 different solvent systems (Fig. 2*a*). The degree of coupling was found to range from 40 to 60 moles of Dox per mole of Mab 1H10 F(ab')$_2$, 25%-oxidized dextran being used as a linker. Attempts to increase Dox loading by increasing dextran oxidation resulted in conjugates with reduced antibody activity (data not shown). 1H10-Dox appeared as a major band after separation by non-reduced SDS-PAGE with an estimated molecular weight of 170 kDa (Fig. 2*b*). 1H10-Dox retained antigen-binding activity to CaSki cervical carcinoma cells (Fig. 2*c*). In contrast, 12.8-Dox, a control antibody-doxorubicin conjugate, did not bind CaSki cells.

In vitro *activity of 1H10-Dox*

The pharmacological activity of 1H10-Dox *in vitro* was assessed by measuring inhibition of cellular RNA synthesis. Incubation of antigen-positive CaSki cells with 1H10-Dox for 20 hr resulted in a dose-dependent inhibition of [5-^{3}H]-uridine incorporation (Fig. 3*a*) with a toxic effect equal to incubation with doxorubicin alone (IC_{50} = 24 and 28 ng/ml, respectively). 1H10-Dox killing of CaSki cells was antibody-mediated, as shown by the lower toxicity of 12.8-Dox, a control MAb-Dox conjugate unable to bind to CaSki cells (IC_{50} = 715 ng/ml) and the low toxicity of doxorubicin conjugated to dextran (IC_{50} = 3550 ng/ml). Further evidence of selective killing of cervical carcinoma cells *in vitro* by 1H10-Dox is provided by the low toxicity of 1H10-Dox to H2669 melanoma cells, a control cell line not bound by MAb 1H10 (Fig. 3*b*). *In vitro* cytotoxicity results, as well as treatment sensitivities and selectivities (Roffler *et al.,* 1991*b*), are summarized in Table I. 1H10-Dox was about 25 times more selective for CaSki cervical carcinoma cells than for H2669 melanoma control cells.

Internalization of MAb 1H10 and 1H10-Dox

The ability of MAb 1H10 and 1H10-Dox to internalize into CaSki cells was examined by fluorescent microscopy. CaSki cells incubated with MAb 1H10 IgG at 4°C and subsequently reacted with goat anti-mouse IgG-FITC conjugate showed strong surface immunofluorescence (Fig. 4 *a*2). In contrast, cells that were incubated at 37°C for 30 min prior to labeling with anti-mouse FITC conjugate exhibited reduced surface fluorescence, indicating that surface-bound MAb 1H10 internalized into CaSki cells and was unavailable for labeling with FITC conjugate (Fig. 4 *b*2).

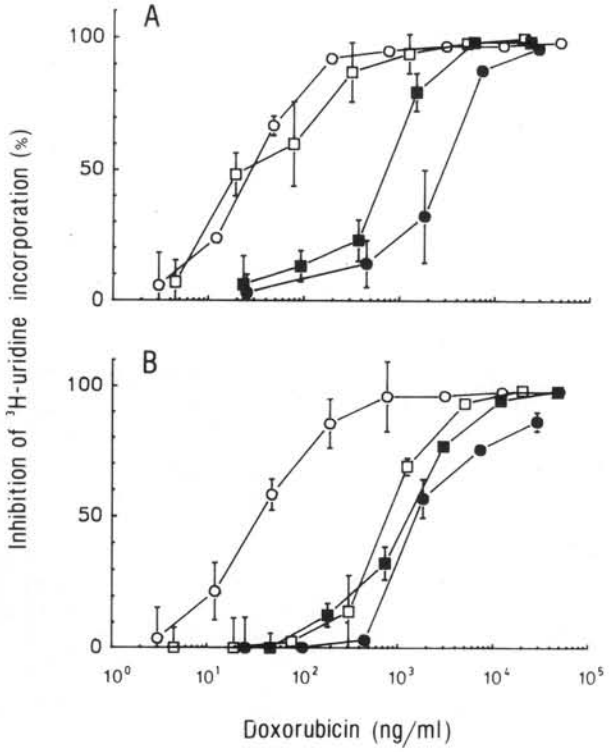

FIGURE 3 – *In vitro* inhibition of tumor-cell RNA synthesis by doxorubicin and doxorubicin conjugates. Inhibition of [5-^{3}H]-uridine incorporation in *(a)* CaSki and *(b)* H2669 cells treated with Dox (○), 1H10-Dox (□), 12.8-Dox (■) or dextran-Dox (●) for 16 hr. Results are expressed as % inhibition of [5-^{3}H]-uridine incorporation in treated cells compared with untreated control cells, and represent the mean of 3 determinations. Error bars show the standard errors of the mean.

To further verify internalization of MAb 1H10, surface antigen on CaSki cells was first saturated with MAb 1H10 F(ab')$_2$-FITC conjugate at 4°C. Cells were then incubated at 4°C or 37°C and surface-bound MAb 1H10 F(ab')$_2$-FITC was subsequently removed by competition with excess unlabeled MAb 1H10. Excess MAb 1H10 reduced the fluorescence of cells continually held at 4°C (Fig. 4 *c*2) as compared with that of cells incubated at 37°C (Fig. 4 *d*2). This result indicates that at 37°C MAb 1H10 F(ab')$_2$-FITC was internalized into CaSki cells where it was inaccessible for competition with unlabeled MAb 1H10.

Internalization of 1H10-Dox was examined by directly monitoring the fluorescence of Dox in the conjugate. CaSki cells were incubated at 37°C in the presence of 1H10-Dox to allow internalization of the conjugate. After surface-bound 1H10-Dox was removed by competition with excess MAb 1H10 F(ab')$_2$, fluorescence of internalized 1H10-Dox was apparent (Fig. 5 *a*2). Control CaSki cells incubated at 4°C in the presence of 1H10-Dox exhibited reduced fluorescence after competition with MAb 1H10 (Fig. 5 *b*2).

Biodistribution of 1H10-Dox

Groups of 3 nude mice bearing established CaSki human cervical carcinoma tumors were injected i.v. with ^{125}I-labeled 1H10-Dox and blood and organs were removed after 1, 4, 12, 24, 48, 72 and 120 hr and counted for radioactivity. Figure 6*a* shows the distribution of 1H10-Dox in blood, tumor and normal tissues at 4, 12, 24 and 48 hr after administration.

TABLE I – *IN VITRO* SELECTIVITY OF 1H10-DOX FOR CASKI CERVICAL CARCINOMA CELLS

Treatment	CaSki		H2669		Selectivity CaSki/H2669
	IC_{50}	Sensitivity	IC_{50}	Sensitivity	
Doxorubicin	28	1.0	35	1.0	1.0
1H10-Dox	24	1.17	760	0.046	25.4
12.8-Dox	715	0.039	1270	0.028	1.41
Dextran-Dox	3550	0.0079	1510	0.023	0.34

IC_{50} values were determined by interpolation from Fig. 3 and were normalized for the amount of doxorubicin in conjugates. Sensitivity of cells to treatments was calculated by sensitivity = $(IC_{50})_{Dox}/(IC_{50})_{treatment}$. Selectivity of treatments for CaSki cervical carcinoma cells compared with H2669 control cells was calculated by selectivity = $sensitivity_{CaSki}/sensitivity_{H2669}$.

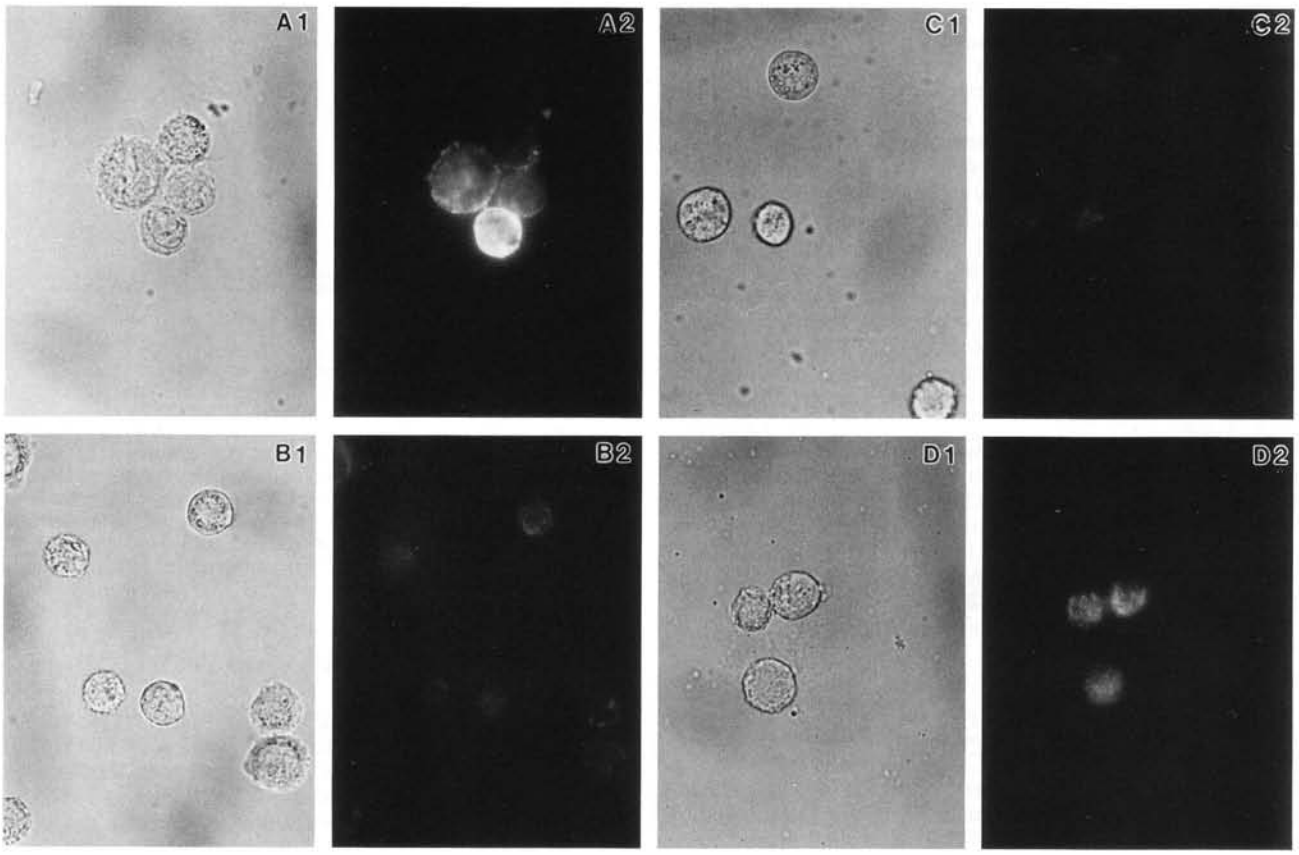

FIGURE 4 – Internalization of MAb 1H10. Internalization of MAb 1H10 into CaSki cells was examined by indirect *(a, b)* or direct *(c, d)* immunofluorescence. In the indirect method, live CaSki cells were incubated with MAb 1H10 at 4°C, washed, incubated at 4°C *(a)* or 37°C *(b)* for 30 min, and probed with FITC-conjugated goat anti-mouse IgG. In the direct method, live CaSki cells were incubated with FITC-conjugated MAb 1H10 at 4°C for 1 hr, washed, incubated at 4°C *(c)* or 37°C *(d)* for 30 min and then incubated twice with MAb 1H10 ascites to remove surface-bound FITC-conjugated MAb 1H10. Cells were observed under phase contrast (1) or fluorescent (2) light.

1H10-Dox accumulation in tumors was rapid, reaching a maximum of 400 cpm/mg tumor after 24 hr, corresponding to 6.3 μg 1H10-Dox per g tumor. In contrast, 1H10-Dox cleared rapidly from the blood, following apparent first-order kinetics during the first 24 hr, with a half life of 3.7 hr. Clearance from heart, spleen and kidney was also rapid, with half lives of 4.9, 5.6 and 4.2 hr respectively. 1H10-Dox clearance from lung and liver were slower, with half lives of 9.9 and 9.4 hr. Tumor-to-non-tumor ratios of 1H10-Dox generally increased throughout 24 hr, reaching a maximum at 24 to 48 hr, due to the slower clearance of 1H10-Dox from tumor than from blood and normal tissues (Fig. 6*b*). By 24 hr post-injection, tumor/tissue ratios of 1H10-Dox ranged from 17 to 50 for blood, heart, lung, bone and gut tissue. Tumor/tissue ratios of 1H10-Dox were lowest in liver and bladder tissues after 24 hr, at between 4 and 5.

In vivo *activity of 1H10-Dox*

The *in vivo* anti-tumor activity of 1H10-Dox was evaluated in nude mice bearing solid CaSki human cervical carcinoma xenografts. Therapy of mice was initiated 5 days after injection of tumor cells to allow establishment of solid tumors. Groups of 5 mice were treated 8 times every 3 or 4 days to maintain a high concentration of Dox at the tumor site, since biodistribution studies showed prolonged localization of 1H10-Dox for 48 to 72 hr.

In the first experiment, the effect of 1H10-Dox dose on tumor inhibition and mouse survival was studied. Doses of

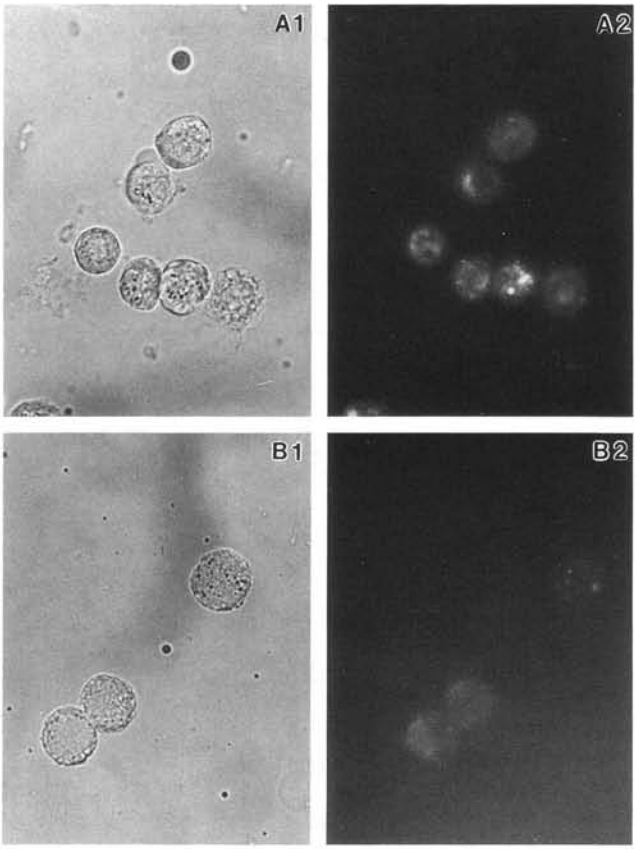

FIGURE 5 – Internalization of 1H10-Dox. Live CaSki cells were incubated with 1H10-Dox for 2 hr at 37°C *(a)* or 4°C *(b)*, washed and incubated with excess MAb 1H10 IgG to remove surface-bound 1H10-Dox. Cells were then observed under phase contrast (1) or fluorescent (2) light.

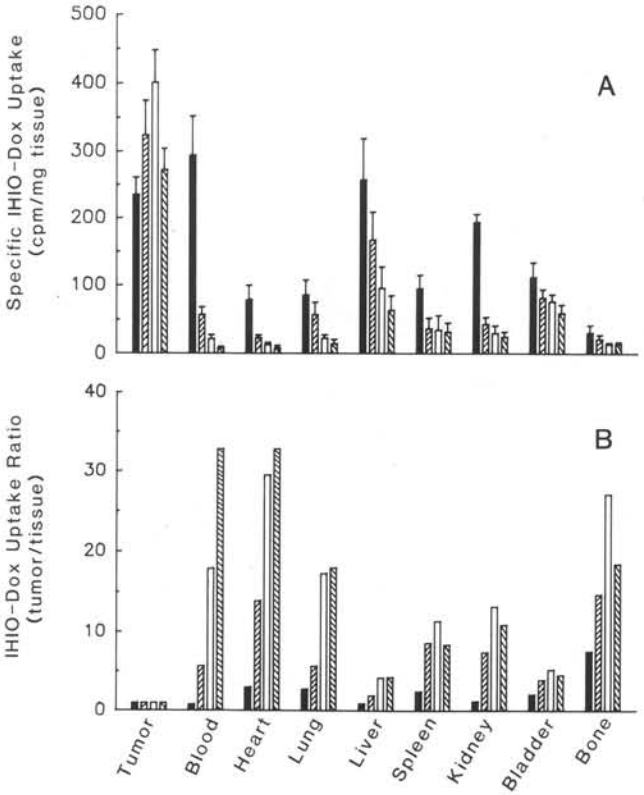

FIGURE 6 – Biodistribution of ^{125}I-labeled 1H10-Dox in athymic mice bearing human cervical carcinoma tumors. Nude mice bearing established CaSki tumors were injected with 20 μg ^{125}I-labeled 1H10-Dox at time zero. Tumors and tissue from groups of 3 mice were removed at 4 hr (■), 12 hr (▨), 24 hr (□) or 48 hr (▧) and specific 1H10-Dox uptake *(a)* or the ratio of 1H10-Dox uptake in CaSki tumors to that in non-tumor tissue *(b)* were determined. Results represent mean values of 3 mice; standard errors are indicated.

1H10-Dox equal to or more than 120 μg (corresponding to 20 μg of conjugated Dox) effectively suppressed cervical carcinoma tumor growth (Fig. 7*a*). Multiple injections of 1H10-Dox containing 40 or 60 μg of conjugated Dox, however, resulted in animal weight loss and death (Fig. 7*b*). In contrast, mouse weight was stable after receiving multiple injections of 1H10-Dox containing 20 μg of conjugated Dox. A dose of 1H10-Dox containing 20 μg of conjugated Dox per injection, equivalent to about 120 μg MAb 1H10 $F(ab')_2$, was used in subsequent experiments.

In the second experiment, mice bearing CaSki cervical tumors received 8 i.p. injections of 1H10-Dox or control treatments. Fig. 8*a* shows that by day 22, mean tumor size in mice treated with 1H10-Dox was 73 mm^3, significantly smaller than tumors in control mice receiving 100 μg injections of human serum albumin (798 mm^3, $p < 0.05$). Average V_t/V_c ratio, determined from tumor volumes of treated and control mice, was 0.021 on day 40. Average tumor sizes in mice receiving 100-μg injections of MAb 1H10 $F(ab')_2$ or 120 μg injections of control immunodrug 12.8-Dox, in contrast, were similar to control mice treated with human serum albumin ($V_t/V_c = 0.75$ and 0.69 respectively), indicating that 1H10-Dox suppression of tumor growth was due to specific targeting of Dox to tumor cells. Eight injections of 20 μg Dox also had little effect on tumor growth; V_t/V_c was still 0.59 on day 40. Even though Dox treatment did not effectively suppress tumor growth, the average weight of these mice was significantly less than that of control mice (Fig. 8*b*). Mean weight of Dox-treated mice was 19.4 g on day 40, compared with 28.0 g for human-serum-albumin-treated mice ($p < 0.005$), indicating that Dox was toxic to mice. Mean body weight of 1H10-Dox-treated mice on day 40 (25.7 g) was less than that of control mice (28.0 g) but similar to 1H10 $F(ab')_2$- and 12.8-Dox-treated mice (25.9 and 25.5 g respectively).

A third *in vivo* experiment was performed to examine the effect of 1H10-Dox administration route on treatment efficacy. In addition, a control group was included in which mice received a mixture of unconjugated Dox and MAb 1H10 $F(ab')_2$. This group was added to control for non-specific synergy between free Dox and antibody. Figure 9*a* shows that i.p. and i.v. injections of 1H10-Dox were equally effective at suppressing CaSki tumor growth; after day 29, tumors in 1H10-Dox-treated mice were significantly smaller ($p < 0.05$) than in mice receiving human serum albumin. By day 47, V_t/V_c ratios were 0.031 and 0.032 for mice treated i.v. or i.p. with 1H10-Dox. A combination of unconjugated Dox and MAb 1H10 $F(ab')_2$ suppressed tumor growth no better than Dox alone. V_t/V_c was 0.55 on day 42 for combined drug and antibody, compared with 0.59 on day 40 for treatment with drug alone. In agreement with previous experiments, both i.p. and i.v. administration of 1H10-Dox affected mouse body weight less than did mixtures of antibody and doxorubicin (Fig. 9*b*).

DISCUSSION

In this study, doxorubicin was conjugated to MAb 1H10 $F(ab')_2$ via dextran linkers to form an anti-cervical carcinoma

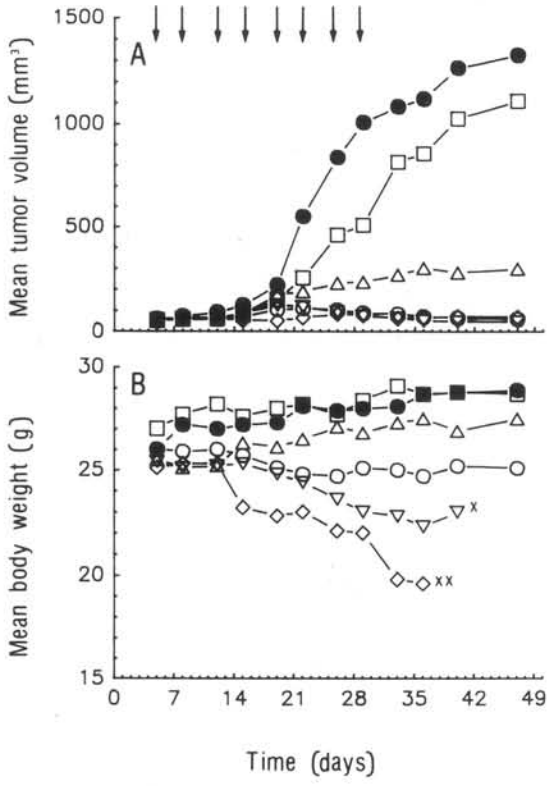

FIGURE 7 – Determination of optimum dose for *in vivo* suppression of cervical tumor growth by 1H10-Dox. Athymic mice bearing established CaSki cervical carcinoma xenografts were treated with 8 i.p. injections (arrows) of 100 μg human serum albumin (●), 120 μg MAb 1H10 F(ab')$_2$ (□), or 60 μg (△), 120 μg (○), 240 μg (▽) or 360 μg (◇) 1H10-Dox. *(a)* Mean tumor volume and *(b)* mean weight per mouse of groups of 5 mice are shown. Mouse deaths are also indicated (×).

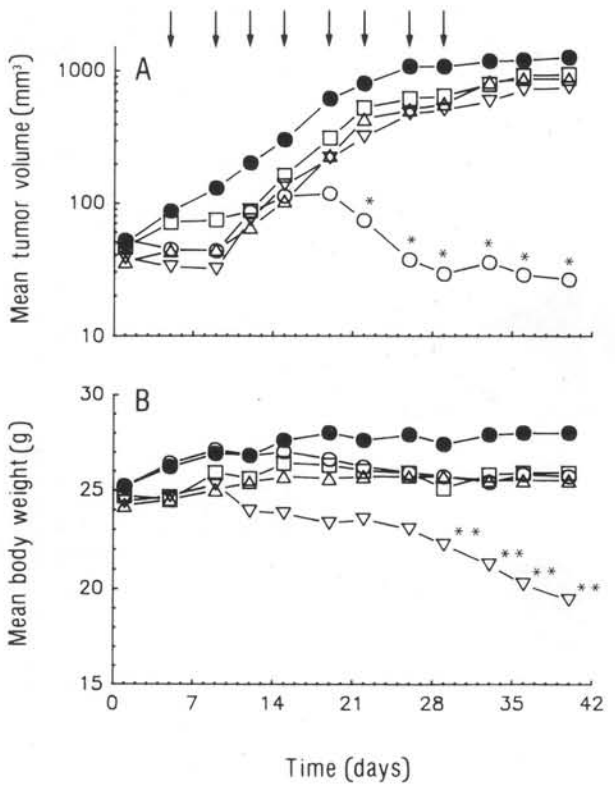

FIGURE 8 – Tumor suppression of established cervical carcinoma xenografts by 1H10-Dox. Nude mice bearing CaSki tumors were treated with 8 i.p. injections (arrows) of 100 μg human serum albumin (●), 100 μg MAb 1H10 F(ab')$_2$ (□), 20 μg doxorubicin (▽), 120 μg 12.8-Dox (△) or 120 μg 1H10-Dox (○). 12.8-Dox and 1H10-Dox concentrations were adjusted to provide 20 μg of conjugated Dox per injection. *(a)* Mean tumor volumes and *(b)* mean body weights of groups of 5 mice are shown. Significant differences between human serum albumin-treated and test mice are shown (*, $p < 0.05$; **, $p < 0.005$).

immunodrug. We were able to achieve a molar ratio of drug/antibody of 40 to 60 without loss of antibody binding activity. 1H10-Dox effectively suppressed RNA synthesis of cervical carcinoma cells *in vitro* and the growth of established human cervical carcinoma xenografts in athymic mice. 1H10-Dox was as effective as free Dox *in vitro* and was more effective and less toxic than doxorubicin *in vivo.*

MAb 1H10 was used to target Dox to cervical carcinoma tumors because this antibody has been shown to react with a membrane antigen expressed on the surface of about 40% of human cervical tumors without normal tissue binding (Roffler *et al.,* 1991*a*). This antibody, when linked with *Pseudomonas* exotoxin, a protein capable of inhibiting protein synthesis in eucaryotic cells by ADP-ribosylating elongation factor 2, has also been shown to effectively kill human cervical xenografts in athymic mice (Roffler *et al.,* 1991*b*). The F(ab')$_2$ fragment of MAb 1H10 was used because it has been shown that, compared with whole IgG, antibody fragments have shorter circulation half-lives (Wahl *et al.,* 1983; Buchegger *et al.,* 1990), better tumor penetration (Buraggi *et al.,* 1985; Vacca *et al.,* 1988) and are probably less immunogenic in humans (Shawler *et al.,* 1985).

1H10-Dox was able to specifically inhibit RNA synthesis of CaSki cervical carcinoma cells *in vitro.* Specificity was shown by the greater inhibition of 1H10-Dox to CaSki cells compared with Dox linked to dextran or 12.8-Dox, a control immunodrug unable to bind to CaSki cells. 1H10-Dox was also about 25 times less toxic to H2669 melanoma cells, a cell line that MAb 1H10 does not bind.

Although Dox can kill cells without being internalized (Triton and Yee, 1982), our results suggest that for maximum activity, 1H10-Dox must be internalized by CaSki cells. 1H10-Dox was able to inhibit CaSki cell RNA synthesis as well as unconjugated Dox, suggesting that 1H10-Dox entered the cells. In addition, doxorubicin linked to dextran, which was in intimate contact with cells for 16 hr during *in vitro* cytotoxicity tests but would be expected to be internalized by cells only slowly, was over 100 times less toxic to CaSki cells than was 1H10-Dox. 1H10-Dox was also less toxic to H2669 cells than CaSki cells, even though 1H10-Dox was also in contact with both cells for 16 hr during the *in vitro* assay. Results from internalization studies are also consistent with an internal target for 1H10-Dox. Fluorescent-labeled MAb 1H10 F(ab')$_2$ was able to enter CaSki cells in physiological conditions. In addition, fluorescence was also observed in CaSki cells after incubation with 1H10-Dox. Although we cannot rule out the possibility that fluorescence observed inside CaSki cells after incubation with 1H10-Dox was due to free Dox dissociated from 1H10-Dox, this seems unlikely, due to the stability of MAb 1H10-Dox both in aqueous and in organic solvents. Taken together, our results suggest that 1H10-Dox must be internalized to exert its full cytotoxic activity. Our results do not preclude the possibility that membrane damage is also important in 1H10-Dox cytotoxicity, nor do they elucidate the actual mechanism of 1H10-Dox cell killing. Further work is under progress to determine the routing of 1H10-Dox in CaSki cells and its mechanism of cytotoxicity.

Biodistribution studies using isotope-labeled immunodrug demonstrated that 1H10-Dox localized preferentially in CaSki tumor xenografts in nude mice. Maximum accumulation of

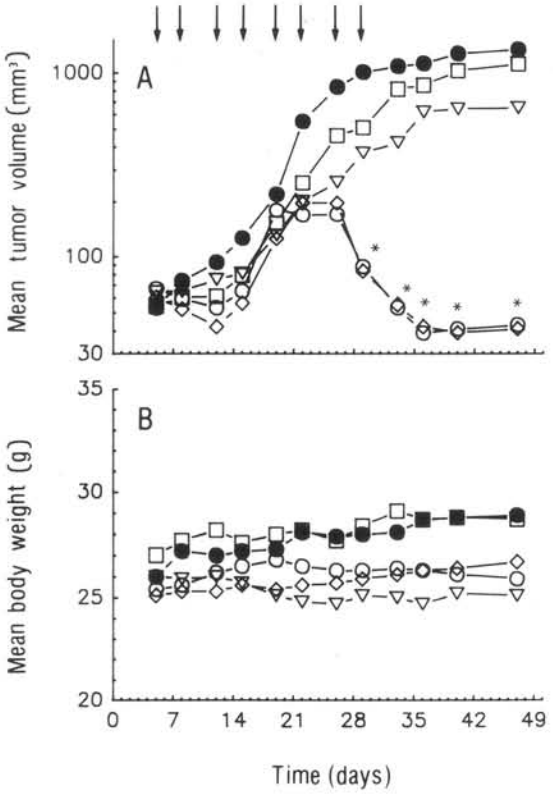

FIGURE 9 – Effect of route of 1H10-Dox administration on tumor suppression efficacy. Nude mice bearing subcutaneous CaSki tumors were treated with 8 i.p. injections (arrows) of 100 μg human serum albumin (●), 100 μg MAb 1H10 F(ab')$_2$ (□), 20 μg Dox and 100 μg MAb 1H10 F(ab')$_2$ (▽), 120 μg 1H10-Dox (○) or 8 i.v. injections of 120 μg 1H10-Dox (◇). 12.8-Dox and 1H10-Dox concentrations were adjusted to provide 20 μg of conjugated Dox per injection. *(a)* Mean tumor volumes and *(b)* mean body weights of groups of 5 mice are shown. Significant differences between human-serum-albumin-treated and test mice are shown (*, $p < 0.05$).

1H10-Dox in tumor tissue occurred about 24 hr after i.v. injection of 20 μg of conjugate containing 4 μg conjugated Dox. An average of 6.3 μg 1H10-Dox per g tumor mass accumulated in xenografts after 24 hr. 1H10-Dox accumulation in xenografts was also prolonged with about 0.8 μg doxorubicin per g tumor remaining after 48 hr, assuming no dissociation of Dox from the conjugate. The prolonged accumulation in tumor tissue resulted in high tumor/tissue ratios from 12 to 72 hr after injection of 1H10-Dox.

1H10-Dox distribution in the blood pool was assumed to be almost immediate, since conjugate was introduced by i.v. injection. Clearance of 1H10-Dox from blood and heart tissue was rapid, following apparent first-order kinetics during the first 24 hr after administration, with half-lives of 3.7 and 4.9 hr respectively. Tumor/tissue ratios of 1H10-Dox in blood and heart tissue were high, reaching 17.9 and 29.5 after 24 hr. This result, along with our *in vitro* results indicating that 1H10-Dox is less toxic to cells if not internalized, suggests that cardiotoxicity, the major dose-limiting toxicity of doxorubicin (Lefrak *et al.,* 1973; Minow *et al.,* 1977), may be reduced by the use of 1H10-Dox.

The anti-tumor effect of 1H10-Dox was evaluated *in vivo* against established solid human cervical carcinoma xenografts in athymic mice. Treatment of established solid tumors was examined to create an experimental situation in which 1H10-Dox would have to pass through anatomical barriers before binding to tumor cells. The optimum dose of 1H10-Dox when given twice a week for a total of 8 i.p. injections was 120 μg conjugate containing 20 μg doxorubicin, corresponding to an accumulated dose of 160 μg Dox. Further *in vivo* studies showed that 8 injections of 1H10-Dox conjugate containing 20 μg Dox effectively suppressed the growth of established cervical tumors throughout the course of the study (49 days). 1H10-Dox suppression of tumor growth was more effective and less toxic than treatment with free Dox. Treatment of tumor-bearing mice with an accumulated dose of 160 μg Dox caused significant toxicity, as shown by mouse death and weight loss. The greater efficacy and lower toxicity of 1H10-Dox is in agreement with results of the biodistribution study showing selective accumulation of 1H10-Dox in tumor tissue and rapid clearance from the heart and blood pool.

1H10-Dox suppression of tumor growth *in vivo* was specific, as shown by ineffective tumor suppression in mice treated with 12.8-Dox, a control IgG_3 F(ab')$_2$-doxorubicin conjugate unable to bind to cervical tumor xenografts. In addition, MAb 1H10 F(ab')$_2$ alone or in combination with free Dox also failed to significantly suppress cervical tumor growth. Tumor suppression by 1H10-Dox was also independent of the route of immunodrug administration; tumors were suppressed equally well in mice receiving 1H10-Dox by either i.p. and i.v. routes. These results indicate that 1H10-Dox was able to specifically recognize antigens present on the surface of subcutaneous cervical tumors in nude mice and accumulate in the tumor to a degree sufficient for tumor suppression.

Others have coupled doxorubicin or the closely related analog daunorubicin to MAbs and studied their anti-tumor effects *in vivo.* Aboud-Pirak *et al.* (1989) also used a dextran linker to conjugate a very high 82 Dox molecules per anti-epidermal growth factor receptor MAb IgG_{2a}. They were able to show reduced growth rate of small human epidermoid carcinoma xenografts in nude mice after administration of a total of 100 μg of linked Dox in 3 i.v. doses, starting one day after tumor implantation. Yang and Reisfeld (1988) used an acid-labile *cis*-aconitic anhydride linker to form conjugates containing 10 Dox molecules per MAb 9.2.27, an IgG_{2a} antibody against proteoglycans expressed on human melanoma cells. They found that the growth of human melanoma xenografts in nude mice was retarded by 10 i.v. injections of conjugate corresponding to a total of 75 μg Dox throughout the experiment, although the average tumor size of immunodrug-treated tumors on day 40 was 10 times larger than at the initiation of treatment. Dillman *et al.* (1988) also used an acid-sensitive *cis*-aconitate anhydride linker to conjugate 25 daunorubicin molecules per anti-CD5 IgG_{2a}. MOLT-4 human T-cell leukemia tumors implanted s.c. in nude mice were suppressed for 3 weeks with only a single i.p. injection of this conjugate containing 30 to 40 μg of daunomycin. The exceptional activity of this conjugate may be related to the high expression and rapid internalization of CD5 on T cells, the antigen target of the conjugate. Many tumor-associated antigens, however, may internalize at a slower rate than those present on lymphoid cells (Sinkule *et al.,* 1991). From the above results and the results presented in this work, larger amounts of drug or more injections are probably required to completely suppress the growth of most solid tumors, suggesting that conjugates containing high Dox-to-antibody ratios are required. The prolonged tumor suppression found in this work may have been due to the large amount of drug that could be targeted and the possible better tumor penetration of the smaller F(ab')$_2$ fragment.

In summary, 1H10-Dox has been shown to preferentially inhibit RNA synthesis of CaSki cervical carcinoma cells *in vitro* and to specifically localize in and suppress the growth of human cervical xenografts *in vivo.* Our results suggest that 1H10-Dox may be useful for the treatment of refractive or metastatic cervical carcinoma lesions in humans.

ACKNOWLEDGEMENTS

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