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Monoclonal antibody L6-daunomycin conjugates constructed to release free drug at the lower pH of tumor tissue

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Summary. Measurements in cancer patients showed that the pH of tumors averages 0.8 unit lower than that of the surrounding normal tissues, confirming published work. Based on this, the anti-carcinoma monoclonal antibody (mAb) L6 was used to prepare immunoconjugates with daunomycin (DM), the drug being released at the acidic pH of the tumor. A direct linking of the aconitic derivative of DM (AcoDM) to mAb L6 led to conjugates that either had a low drug/antibody ratio (<5 : 1) or precipitated in vitro. In order to increase the drug load and avoid precipitation, several biopolymers were tested as spacers between the drug and the L6. To attach the polymer derivative to the mAb, the former was maleimidized and the mAb was thiolated. The AcoM/mAb ratio obtained was 20, and the mAb retained its highly specific binding to tumor cells. At pH 6 the AcoDM-L6 conjugate was toxic to cultured C-3347 carcinoma cells with an inhibitory concentration (IC_{50}) of 5 μg/ml. The conjugate was less effective than the free DM with an IC_{50} of 0.2 μg/ml. The L6 alone was not toxic. At a tumor pH of 6.5, 15% of the AcoDM was released. The amount of released drug reached a maximum 24–48 h after exposure to the acidic medium.

In vivo localization studies demonstrated a similar tumor uptake of the conjugate and mAb L6 with 18% of the injected dose/g tumor and a maximum uptake in tumor 48 h after injection. Our data indicate that it is possible to construct conjugates based on a pH-sensitive linker that can be targeted successfully to a tumor with release of a portion of the drug at the tumor site, but testing is needed to establish whether such release has anti-tumor activity in vivo and offers an advantage over treatment with unconjugated drug.

Key words: L6-daunomycin conjugates – Monoclonal antibody – Drug release

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Introduction

Although various chemotherapeutic drugs have been found to be effective against certain tumors [12, 33], there is a need for therapeutic agents with greater selectivity and efficacy. An attractive approach is to prepare drug-antibody conjugates that can utilize the antibody's ability to bind to tumor cells, in order to target the drug to tumors. Several immunoconjugates of this type have been prepared and shown to (a) be selectively cytotoxic to antigen-positive tumor cells in vitro, (b) localize in tumors in vivo, and (c) have antitumor activity in nude mice that is greater than that of the drug or the antibody alone [6, 24]. However, there has not yet been much therapeutic success in treating human cancer patients with immunoconjugates, and the development of better methods for tumor targeting may be needed.

Most chemotherapeutic drugs must be taken up by tumor cells in order to kill them. An immunoconjugate must, therefore, bind to the cancer cells and then either be internalized by these cells (with active intracellur release of the drug) or liberate the drug in the close vicinity of the tumor cells, so that it can be taken up and act as when used conventionally. The latter approach has several advantages. While free drug can be taken up by most cells, many immunoconjugates are not internalized. Heterogeneity exists in the expression by tumor cells of various tumor antigens, with antigen-negative cells, occurring frequently [34]. Moreover, some tumor antigens, mucins, for example, are present in large amounts in the vicinity of the cancer cells, suggesting the potential for targeting tumor regions. One approach, which leads to the conditional release of free drug in tumor, has been published by Senter et al. [26].

The pH of tumor tissue has been reported to be 0.5–1.0 unit more acidic than that of healthy tissue, in studies of various types of neoplasms [3, 22, 30]. Techniques for the chemical linking of drugs or toxins with antibodies via an acid-sensitive bond have been developed [9, 28]. On the basis of that work, we have attempted to develop methods for preparing immunoconjugates capable of releasing free

drug at a pH 0.5–1.0 unit lower than that of normal tissue. This paper confirms the demonstration of a lower tumor pH and describes the properties of a pH-sensitive conjugate. The conjugate was prepared between daunomycin (DM) and a mouse mAb L6, which binds to cells from most human carcinomas without being internalized (unpublished data) and reacts only very weakly with normal tissues [16].

Materials and methods

Reagents

Daunomycin hydrochloride, polylysine (L and D isomers), polyglutamic acid, polyaspartic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, *cis*-aconitic anhydride and hydroxylamine were purchased from Sigma Co. (St. Louis, Mo.); *S*-acetylmercaptosuccinic anhydride was from Aldrich (Milwaukee, Wis.) and sulfosuccinimidyl-4-(*n*-maleimidomethyl)-cyclohexane 1-carboxylate was obtained from Pierce (Rockford, Ill.).

Cell cultures

Lung carcinoma line L-2981 and colon carcinoma line C-3347 [16] were used as sources of target cells. Cultures were maintained in 75 cm^3 tissue-culture flasks (Falcon Plastics, Oxnard, Calif.) using incomplete modified Dulbecco's medium (IMDM; Gibco, Grand Island, N. Y.) supplemented with 15% heat-inactivated fetal calf serum (Hyclone, Sterile Systems, Logan, Utah), at 37° C, in a humidified atmosphere containing 5% CO_2. After 3–4 days, when the cells had grown to confluency, they were harvested after incubation for 5 min with 0.02% EDTA in phosphate-buffered saline (PBS; Baker Chemicals, Phillipsburg, N. J.) and resuspended in RPMI medium with 15% fetal calf serum. All cultures were free of mycoplasma.

mAbs

Purification of mAb L6 (an IgG2a) was carried out as described previously [17]. Immunoglobulin (IgG2a) from mouse myeloma line pl. 17 was purified on protein A as described previously [17] and used as a control. mAb 1F5 (IgG2a), which recognizes the Bp35 antigen of B cells, was generously provided by Dr. J. A. Ledbetter at Oncogen and served as another control mAb.

pH measurements in cancer patients

Ten patients (8 female and 2 male; mean age 67.3 years) with different types of tumors were studied during surgery. A flexible pH probe, diameter 1.2 mm (Microelectrodes 20142, Microelectrodes Inc., N. H.) connected to a digital pH meter (Beckman model 3500) was inserted into normal and tumor tissues through a 14-gauge needle, and the patient's index finger was connected to a reference electrode (NMI-401, Microelectrodes, Inc.). The probe was calibrated before and after the procedure for each patient. It was sterilized with Turgicos solution (Johnson & Johnson, Arlington, Tex.). The pH values were recorded after stabilization, usually within 5–10 min. Two patients received 50 ml 50% glucose solution i. v., which was given over a 30-min period beginning 1 h before surgery.

Conjugation reactions

Preparation of aconitic daunomycon complex (AcoDM). A protocol described by others [28] was modified as follows: 16 mg DM was dissolved in 1.5 ml ice-cold water, and 16 mg *cis*-aconitic anhydride was added slowly (as a powder) to the dissolved DM. The pH was maintained at 9.0 by the addition of 0.5 M NaOH. The mixture was stirred for 15 min, the pH was then adjusted to 3 by cold HCl, and the reaction solution was stirred at 4° C for 15 min.

The AcoDM was isolated from the pellet by centrifugation for 10 min at 4° C at 1000 *g*. The pellet was resuspended in 1 ml PBS and the pH adjusted to approximately 8. Alternatively, the AcoDM was prepared by adding to the daunomycin solution the *cis*-aconitic anhydride dissolved in dioxane. The proportion of conversion of DM to AcoDM was estimated by thin-layer chromatography using a solvent mixture of acetone : chloroform : acetic acida (17 : 3 : 1). Under these conditions, the R_F of the free drug was ≈0.1 and that of AcoDM was 0.5. Using the above-described procedure, only one spot of AcoDM was observed.

Direct conjugation of AcoDM to L6 was performed via two pathways. (a) A sample of 0.6 ml AcoDM (10 mg/ml) was added to 1 ml mAb L6 (10 mg/ml PBS, pH 7). Subsequently, 10 mg ethyl-3-(3-dimethylaminopropylcarbodiimide (EDC) was introduced, and the mixture was incubated for 3 h at 4° C at pH 7. The solution as then loaded on a Sephadex G-50 column (38 × 1.8 cm), and 1-ml fractions were collected using PBS eluant. The drug-antibody conjugate emerged in fractions 16–17 and the free DM peaked in fractions 35–42. The complexed AcoDM yield of this conjugation was 7%–10%. The conjugate was further dialyzed against PBS to remove any free drug. (b) Modification using succinylated AcoDM was carried out by adding 10 mg *N*-hydroxysuccinimide and 5 mg EDC to 4 ml AcoDM solution and stirring at room temperature for 24 h (pH 5). The succinylated intermediate of the drug was then linked to the antibody by adding 1 ml succinylated AcoDM to 1 ml mAb L6 (5 mg/ml in PBS buffer) and adjusting the pH to 8.5 with 1 M NaOH, after which the immunoconjugate was isolated as described above for the L6-AcoDM conjugate. This protocol led to a high yield of conjugated Aco-DM, 70% being recovered.

Indirect linking of AcoDM to the antibody via polylysine spacer. A DM-poly(Lys)-mAb immunoconjugate was prepared using a modification of previously described conjugation methods [18, 21].

Thiolation of mAb L6 (L6-SH). The pH of the mAb L6 solution (10 mg/ml) was adjusted to 6.5, after which 40 µl S-acetylmercaptosuccinic anhydride solution (14 mg/0.1 ml dimethylformamide freshly dried over 3A molecular sieves), was added dropwise to the mAb. Thiolation was continued for 30 min at 25° C, after which 0.1 ml 0.1 M Tris/HCl, pH 7, 10 µl 0.1 M EDTA pH 7, and 0.1 ml 1 M hydroxylamine, pH 7 were added. The mixture was incubated for 5 min at 30° C and then loaded on a Sephadex G-25 column (25 × 1.8 cm), which had been washed with a 0.1 M phosphate buffer, pH 6, containing 5 mM EDTA. The peak fractions of the modified mAb were collected, pooled and concentrated to a volume of 0.3 ml. The number of SH groups introduced into the mAb was measured using a modification of the Ellman assay [11, 18].

Attachment of AcoDM to poly(L-lysine). Polylysine was chosen for this study because of its potential to carry large numbers of drug molecules and also in view of its intrinsic ability to inhibit tumor growth and to aid in the transport of both low-molecular-mass compounds and macromolecules into tumor cells [25, 27]. A 0.1 ml sample Aco-DM solution was added to 1 ml poly(L-lysine) 10 mg/ml, with a molecular mass ≈53–59 kDa, at a molar ratio of AcoDM: poly(Lys) of 29, after which 20 mg carbodiimide (EDC) was added to the reaction mixture. The pH was maintained at 7.2. The mixture was stirred for 20 h at 4° C. The drug-polymer complex was then purified on a Sephadex G-50 column (15 × 1.8 cm) using PBS solution as an eluant, and 1-ml fractions were collected. Approximately 70%–80% of the AcoDM was recovered as a complex with the polymer.

Maleimide reaction on poly(Lys) AcoDM. A 25-µl aliquot of sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate of (*S*-SMCC) reagent (17 mg/52 µl dimethylformamide) was added dropwise (very slowly) to 1 ml poly(Lys)-AcoDM complex (pH 7.2). After 30 min incubation at 30° C, the mixture was loaded onto a Sephadex G-25 column (12 × 1.8 cm) and eluted with PBS. Fractions containing poly(Lys)-

AcoDM were pooled and concentrated to a volume of 0.6 ml. The level of maleimide incorporation to the polymer was determined using the assay of Grassetti and Murray [15].

The maleimide derivative of poly(Lys)-AcoDM (0.6 ml) was added slowly to the thiolated mAb (0.3 ml), and the pH was adjusted to 6.2. Nitrogen was then passed into the mixture for 3 min and the mixture was incubated for 1 h at 30° C in a sealed tube with 3 mg 2-ethylmaleimide added to block possible excess-SH groups on the antibody. The L6-poly(Lys)-AcoDM conjugate was purified by precipitation with saturated (55%) ammonium sulfate solution (pH 7.5) for 30 min at 4° C, followed by centrifugation at 9000 *g* for 10 min at 4° C. The pellet was resuspended in 0.5 ml PBS and the pH was adjusted to 7.5. To remove the ammonium sulfate salt, the solution was dialyzed against PBS. The purified conjugate was kept at –20° C until use. A conjugate using a control immunoglobulin (Pl.17 or mAb 1F5) was prepared similarly.

Attempts were made alternatively to purify the conjugate by size-exclusion, protein A and hydroxyapatite chromatography. As a modification, the ammonium sulfate step was sometimes followed by a twofold concentration of the reaction solution, prior to the centrifugation step. Alternatively we tried to maleimidize the mAb and thiolate poly(Lys)-AcoDM. The purity of the conjugates was tested by Paragon gel, and 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All antibody preparations were pyrogen-free.

Aliquots of the isolated conjugate were tested for binding to tumor cells and for cytotoxicity. Quantitative analyses were performed on the conjugate to determine the amounts of MAb by the Bradford assay [5] and the ultraviolet absorption at 280 nm, of poly(L-lysine) [29] and of AcoDM, Testing for AcoDM was based on the absorbance at 475 nm, 1 mg/ml Aco-LDM, having an $A_{475} = 20$).

Binding assays

The purified conjugates were tested for binding to tumor cells, using immunohistology on frozen sections [13].

When poly(Lys) was used as a spacer in the conjugation, the slides were pre-coated with polymer to minimize non-specific binding. Binding assays were performed also on intact tumor cells using a fluorescence-activated cell sorter (FACS with a Coulter Epics model A) and measuring the ability of an immunoconjugate compared to intact L6, to compete with fluorescein-isothiocyanate(FITC)-conjugated L6 for the binding to antigen-positive cells. The assay was done by plating cells in 96-well plates at 10^6 cells/well, followed by washing of the cells. Samples at various dilutions (concentration range: 5–10 ng/ml) were added with FITC-L6, 10 ng/ml, and incubated with target cells at 4° C for 30 min. The cells were washed twice, and transferred to polypropylene tubes for analysis on the FACS. Competitive binding was determined by the percentage of conjugate bound as compared with native mAb L6.

Fixed M-2669 melanoma cells and C-3347 carcinoma cells were diluted to 1×10^7 Cells/ml in RPMI medium +15% fetal calf serum and an aliquot of 1×10^6 cells (100 µl) was added to each 12-mm ×75-mm polypropylene test-tube. The cells were washed twice in 2 ml PBS and centrifugated for 1 min. ^{125}I-labeled mAb was diluted to 10 µg/ml RPMI) medium +15% fetal calf serum at pH levels of 5.5, 6.0, 6.5 and 7.2. The pH of the RPMI medium was adjusted to the desired level just prior to use. A 100-µl aliquot of ^{125}I-mAb was added to the cells and incubated at room temperature for 30 min. The cells were washed twice with 2 ml PBS. The pellets and the supernatants were counted on a Beckman counter and the percentage binding was determined by the ratio of the radioactivity in the pellet to the total radioactivity.

Cytotoxicity evaluations

Two in vitro assays were used, measuring uptake of tritiated thymidine, and measuring colony formation of plated cells. For the thymidine-uptake assay, cells were plated in 96-well plates (10^6 cells/well) and incubated overnight. After washing the plates twice with IMDM, drug conjugate or mAb was added. This was followed by a 1 h incubation, except for a few experiments where incubation was for 5 h. Subsequently the cells were washed twice, pulsed with 20 µl tritiated thymidine, and incubated at 37° C for 4 h. The plates were then frozen at –20° C, thawed, and harvested. The filters were counted in a Beckman 3701 beta counter. The percentage inhibition was calculated as the decrease in uptake of tritium in treated as compared with untreated cells in the log phase. For the clonogenic assay, suspended cells were counted and divided into aliquots in test-tubes at 10^6 cells in 1 ml/well. This was followed by removal of the samples, washing, and counting of cells exposed only to medium, to evaluate the number of cells lost by the procedure. The concentration of the remaining cells was adjusted accordingly, after which these cells were poured into liquid 3% agar in IMDM, which was layered on top of solid 3% agar placed in 24-well plates. The plates were incubated for 10 days and the number of colonies was determined. A colony was defined as a cluster of ten or more cells. The conjugate was exposed to low pH (6) for 48 h, without the cells, after which the pH was adjusted to 7.2. Then the mixture was applied to the cells. As control, a conjugate was used that was incubated at neutral pH for 48 h before it was added to the cells. Extra controls were AcoDM alone, in two concentrations, and L6 alone.

Localization of immunoconjugates in vivo

The ability of L6-poly(Lys)-AcoDM conjugate to localize in tumors, as compared with that of unmodified L6, was examined in nude mice bearing human tumor xenografts. Two randomized groups of 20 mice each were used. In most cases the C-2981 tumor was implanted bilaterally on the flanks of the mice in order to increase the tumor sample size. The implanted tumors were allowed to grow for 3–4 weeks, by which time they had reached a weight of 50–300 mg.

mAb L6 labeled with ^{125}I by the chloramine T method [4]. Each mouse received about 5 µCi labeled L6 or of labeled conjugate (specific activity 10 µCi/µg) along with either 50 µg unlabeled L6 or 50µg unlabeled conjugate, respectively. In addition, each mouse received a comparable ^{131}I-labeled control mAb, 1F5, along with 50 µg unlabeled 1F5 i.v. At several selected time points, 6–120 h later, four animals from each group were anesthetized, exsanguinated through the orbital plexus, and sacrificed. Tumor, liver, spleen, kidney, and blood were removed, weighed, and counted in a gamma counter capable of differentiating between ^{125}I and ^{131}I. Most results were expressed in terms of percentage injected dose/g (ID/g). This quantitiy was calculated on the basis of the sum of the weights of two pieces of tumors and not on the average. The localization index (LI) was calculated as follows (Eq. 1).

$$\mathrm{LI} = \frac{(\mathrm{ID_s\ antibody/g\ tumor})/(\mathrm{ID_s\ antibody/g\ blood})}{(\mathrm{ID_{ns}\ antibody/g\ tumor})/(\mathrm{ID_{ns}\ antibody/g\ blood})}$$

where $\mathrm{ID_s}$ = percentage of injected dose of specific antibody, and $\mathrm{ID_{ns}}$ = percentage of injected dose of non-specific (control) antibody.

Release of the drug from the conjugate at low pH in a cell-free medium

Two spectroscopic techniques were used to monitor the amount of liberated free drug at various pH values: first, absorbance of supernatants separated from the bound AcoDM was measured at 475 nm; second, a technique was used based on the fluorescence of free and bound drug.

For measurement of absorbance, the bound AcoDM was in the form of either poly(Lys)-AcoDM or IgG-AcoDM. The conjugate was diluted with citrate/phosphate buffers of pH ranging between 5.5 and 7.2; this was followed by incubation of the mixtures at 37° C, and removal of 1-ml aliquots at different times. In order to separate daunomycin that was released from the complexes, conjugates were passed through a Centricon-10 filter (Amicon, Danvers, Me.). The absorbance of the supernatants was then monitored for the presence of free drug (A_{475}). The absorbance was converted to concentration of drug based on $A_{475}{}^{1\ \mathrm{mg/ml}} = 20$.

Experiments based on measurements of fluorescence were done in which 2 ml conjugate was dialyzed against 50 ml PBS for 72 h. Samples

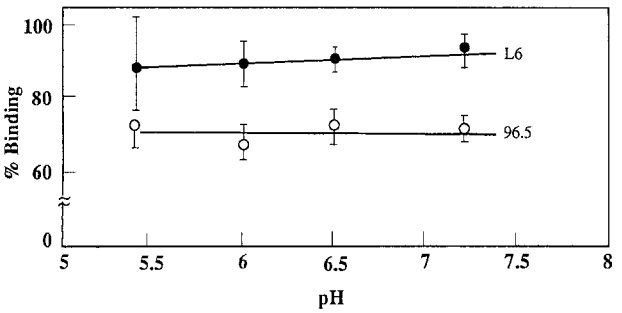

Fig. 1. The effect of pH on the binding of two different mAbs to their respective target cells. The results are expressed as the percentage binding as determined by a cell-binding assay. The average percentage binding for L6 was 86%, and for mAb 96.5 it was 69%. Each point represents an average of three determinations

from the external media were drawn and the amount of daunomycin was measured by fluorescence emission at 560 nm at different times between 2 h and 72 h.

Results

Determination of the pH of tumors

As summarized in Table 1, a significant pH difference between tumor tissue and adjacent normal tissue was consistently observed in the ten cancer patients studied. The pH in the tumor ranged from 6.0 to 6.7, with a mean of 6.4 ± 0.1, compared with a mean pH of 7.2 ± 0.1 in normal tissues. The difference between neoplastic and healthy tissues was 0.5–1.1, with an average of 0.8 pH unit. In patient one, who received an infusion of glucose, the pH gradient reached a value as high as 1.3 units. A low tumor pH was detected in both primary and metastatic lesions and did not appear to depend on tumor size or on whether the pH probe was located superficially or deep inside the tumor. A similar pH difference between normal and neoplastic tissues was noticed also in studies with nude mice transplanted with human carcinoma C-2981 (data not presented).

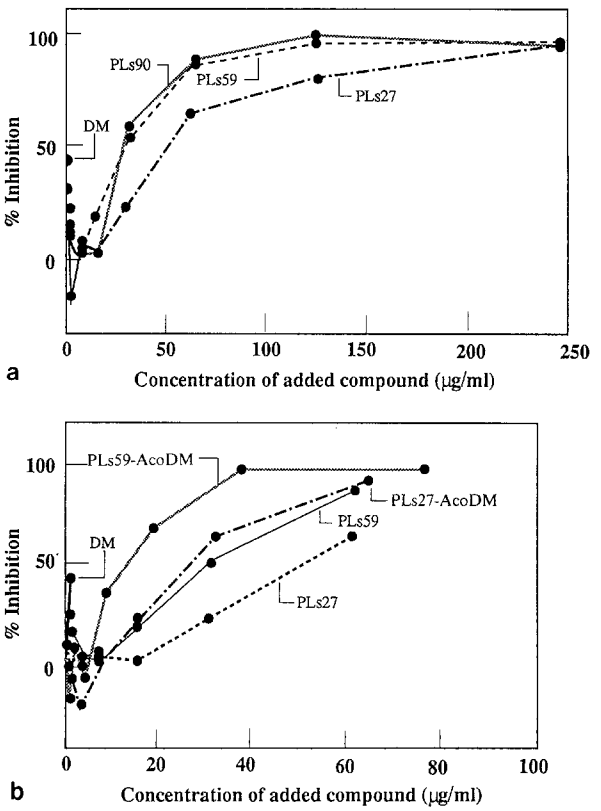

Fig. 2. Inhibition of [^{3}H]thymidine uptake by cells from carcinoma line 3347 exposed to (**a**) poly(Lys) and (**b**) poly(Lys)-aconitic-daunomycin [poly(Lys)-AcoDM] complexes. The data are expressed as percentage inhibition of thymidine incorporation into treated cells as compared with non-treated cells, as a function of the concentration of the added compound

Antibody binding at different pH

The binding of two different mAbs, L6 and 96.5, to cultured target cells was tested at various pH levels (5.5–7.2); mAb L6 binds preferentially to carcinoma cells and mAb 96.5 to melanoma cells, which were the two types of cell used. The binding of mAb to cells did not show any signif-

Table 1. pH Measurements in Tumors and Normal Tissues from Ten Patients

	Age	Sex	Diagnosis	Given i. v. glucose	A pH of normal issue	B pH of tumor tissue	Difference (A-B)
1)	76	F	Cancer of the colon with mets	Yes	7.2 Subc.	5.9	1.3
2)	57	M	Undif. mesenchymal tumor	Yes	7.4 Subc.	6.6	0.8
3)	80	F	Rectal cancer	No	6.9 Pararectal	6.4	0.5
4)	46	F	Mammary cancer	No	7.4 Subc.	6.7	0.7
5)	68	F	Malignant melanoma	No	6.9 Subc.	6.0	0.9
6)	48	M	Lymphoma with axillary mets	No	7.4 Subc.	6.0	0.7
7)	78	F	Cancer of the cardia adenocarcinoma	No	6.9 Subc.	6.0	0.9
8)	77	F	Mammary cancer mets	No	7.1 Subc.	6.5	0.6
9)	76	F	Hypernephroma	No	7.3 Subc.	6.6	0.7
10)	67	F	Cancer of the esophagus	No	7.3 Subc.	6.2	1.1

Mean ± SEM = 7.2 ± 0.1 normal tissue
Mean ± SEM = 6.4 + 0.1 tumor tissue
mets = metastasis

Subc. = subcutaneous
p value = 1.9^{-06}.

icant pH dependence (Fig. 1). There was a mean binding of 86% for mAb L6 and 69% for mAb 96.5.

Inhibition tests of polymer conjugates

As a first step toward developing drug conjugates, we studied some properties of polymers linked to the drug. Figure 2a shows the effect of poly(Lys) on the uptake of tritiated thymidine by cells from the colon carcinoma line C-3347. At a relatively high polymer concentration the uptake was inhibited, to a degree dependent on the molecular mass of the polymer. Hence, poly(Lys) with higher M_r caused an elevated toxicity and the IC_{50} changed from 50 µg/ml [for 27-kDa poly(Lys)] to 30 µg/ml (for the 59-kDa form). The polymer was much less potent than the free drug, which had an IC_{50} of 0.1–0.2 µg/ml as shown in Fig. 2a, b.

The linking of AcoDM to poly(Lys) produced material with a toxicity ranging between the potency of free poly(Lys) and AcoDM (Fig. 2b), with IC_{50} of 15 and 25 µg/ml for the $(Lys)_{59}$-AcoDM and $(Lys)_{27}$-AcoDM, respectively. The L and the D isomers were equally effective. In some cases, the poly(Lys) induced a 3%–15% additional proliferative effect, which was observed at micromolar polymer concentrations.

Conjugation procedures

Direct linking of AcoDM to mAb L6 formed a conjugate with a drug/mAb molar ratio of 3–5, with complete preservation of mAb binding to the target cells. Higher levels of incorporation of the drug damaged antibody binding significantly.

When the AcoDM was attached to the mAb through the succinylated form of the drug, the yield of the conjugation was improved to 20–30 drug molecules/mAb molecule, but the stability in solution of this immunoconjugate was unsatisfactory because of precipitation occurring with time even at 4° C.

This led us to focus on using a spacer as a bridge between the drug and the mAb. The maleimide reagent *S*-SMCC gave better results than other heterobifunctional agents including native SMCC and maleimidobenzoyl-*n*-hydroxysuccinimide ester. The AcoDM/mAb obtained with the spacer poly(Lys) was 18–25 drug molecules/mAb; the poly(Lys)/mAb ratio was 1 : 1. The average number of thiol groups substituted on the poly(Lys) averaged 1.2. This conjugate appeared to be stable for several weeks at low temperatures. When maleimidization of the mAb and thiolation of the polymer were used instead, damage to the mAb was observed.

From the SDS gel electrophoresis studies (data not shown) we conclude that the molecular mass of the l6-poly(Lys)-AcoDM complex is 210–220 kDa. However we occasionally observed a minor band at 300 kDa, which may be attributed to the dimerization of the IgG molecule.

Binding of the conjugate to target cells

The binding of the L6-poly(Lys)-AcoDM immunoconjugate to colon carcinoma cells C-3347 was assayed using

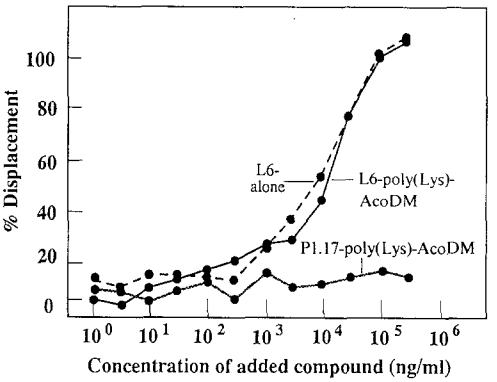

Fig. 3. Competitive binding curves of the immunoconjugates to target cells

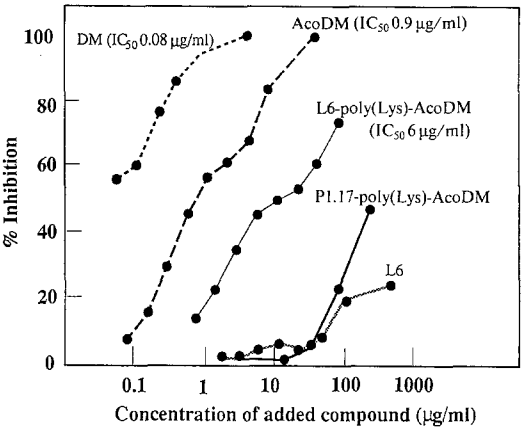

Fig. 4. Inhibition of thymidine incorporation by L6-poly(Lys)-AcoDM conjugate. The results represent one of four similar experiments ($n = 4$)

FITC-L6 as the probe. As shown in Fig. 3, the binding curves of the specific conjugate and the native mAb L6 were superimposed, and a conjugate using the control Pl.17 immunoglobulin did not compete at all with the binding of mAb to the tumor cells. Samples of the conjugates were run on Paragon gel, and formed only one band (data not shown). The position of this band was completely different from that of the bands observed with either unmodified mAb L6 or poly(Lys)-AcoDM. The conjugate migrated toward the negative electrode, indicating that significant change had occurred in the overall charge of the mAb.

Inhibition assays

The toxicity of the L6-poly(Lys)-AcoDM conjugate was tested in several assays. Figure 4 demonstrates the inhibition of thymidine incorporation into carcinoma cells after exposure to the L6-specific conjugate at pH 7. The mean IC_{50} of the drug-L6 conjugate was 5.0 ± 1.5 µ/ml ($n = 7$) for free AcoDM. The non-specific control conjugate was also toxic, but only at 15–20 µg/ml AcoDM present. mAb L6 alone gave no significant inhibition.

In order to compare the toxic effect of conjugate at different pH levels the respective groups were incubated with carcinoma cells for either 1 h or 5 h, followed by

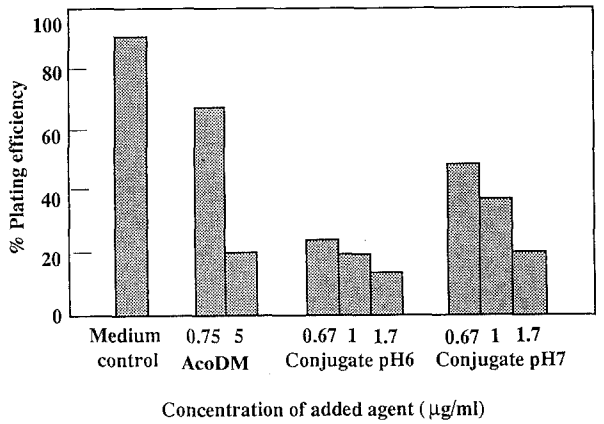

Fig. 5. Colony-inhibition assay measuring the effect of the L6-poly(Lys)-AcoDM at two pH levels. The results are expressed as: inhibition = 100–(number of colonies in treated cells/number of colonies in untreated cells) × 100. These data represent similar results from two other experiments ($n = 4$). The AcoDM concentrations are expressed in μg/ml of drug units, and the conjugates in μg/ml of antibody units. The media were not toxic

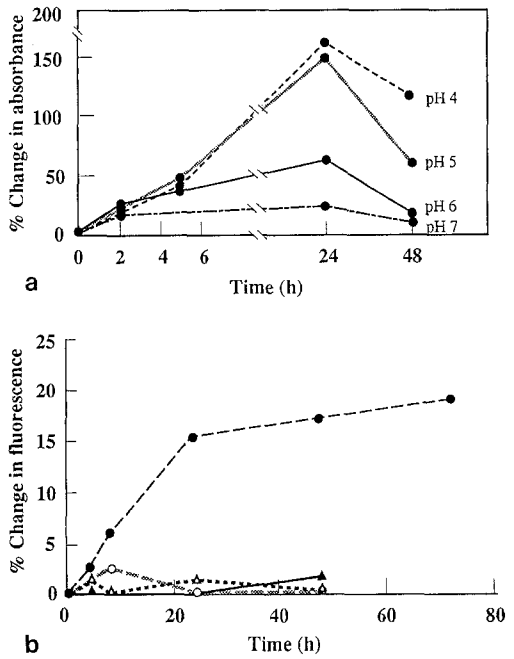

Fig. 6. pH-dependent release of DM with time from (**a**) IgG (absorbance) or (**b**) poly(Lys) (fluorescence). In **a** DM was linked directly to the Ab with a molar ratio of drug/Ab = 3 : 1. The free drug level is expressed as A_{475}. The cell external media consisted of mixtures of citrate/phosphate buffers adjusted to the required pH. **b** The molar ratio of ADM to polymer ≈20 : 1. The amount of liberated drug was calculated from the recorded fluorescence intensity. □, Poly(Lys)-AcoDM, pH 5.7; □, poly(Lys)-AcoDM, pH 7.2; □, poly(Asp)-DM, pH 5.7; □, poly(Asp)-DM, pH 7.2

washing of the cells, and a colony-inhibition assay. The results after a 1-h incubation are summarized in Fig. 5. The data are expressed as the percentage inhibition as compared with the number of colonies in untreated controls. Culture medium alone (at either pH) had a very slight effect, and free AcoDM was toxic in a dose-dependent manner. When the conjugate was exposed to pH 6 followed by normalization to pH 7.2, there was a consistent increase of 20%–25% in colony inhibition, as compared with the numbers obtained when the conjugate was kept at pH 7.2. The effect of the conjugate at low pH was closer to that of free AcoDM when compared on the basis of the concentrations of the two compounds. After 5 h incubation, even higher inhibition levels were recorded (data not presented). Similar observations were obtained by a parallel thymidine-uptake assay (data not presented). Control inhibition experiments were performed with the L6-poly(Lys)-AcoDM conjugate on the L6-antigen-negative melanoma cell line M-2669, at pH 6.0. The inhibition curve was similar to that obtained with the L6-antigen-positive cells C-3347, although the IC_{50} was higher.

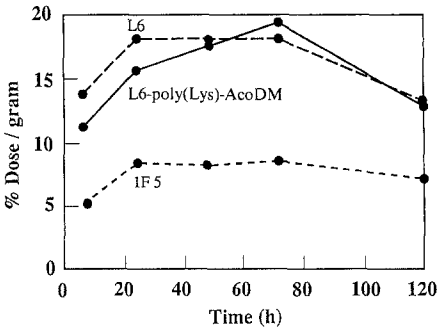

Fig. 7. Tumor uptake of L6 and L6-poly(Lys)-AcoDM conjugate in xenografted nude mice. The results are expressed as percentage injected dose/g tissue vs time after injection (h). Each point represents the average data from three animals. Each mouse was injected with 5 μCi ^{125}I-labeled (L6)native or conjugate), specific activity of 10 μCi/μg, along with 50 μg L6 or conjugate. In addition, each mouse received the same amount of 131-labeled non-specific 1F5 co-administered i. v.

Release of the drug

As shown in Fig. 6a, free AcoDM was liberated from IgG at low pH with the maximum release at 24 h of incubation. Exposure to pH 4 or 5 caused 30%–40% release of AcoDM from the conjugate, while approximately 15% drug dissociation was noted at pH 6. There was no drug release at pH 7.2. The release of AcoDM from poly(Lys) showed the same pH profile as in the AcoDM-IgG system (Fig. 6b). The poly(Asp)-DM complex did not dissociate at either pH 5.7 or 7.2.

Biodistributions in vivo

Data on biodistributions of the labeled mAbs and conjugate are presented in Fig. 7–9. The tumor uptake for both mAb L6 and the L6 poly(Lys)-AcoDM conjugate was similar (Fig. 7). The maximum concentration achieved was 18% ID/g tumor, and the maximum uptake occurred between 48 h and 72 h after injection. The uptake of the control mAb 1F5 in the tumor was significantly lower than that of the specific conjugate or mAb L6 (5% ID/g tumor). Blood clearance was slower with L6-poly(Lys)-AcoDM than for the native L6 (Fig. 8), with 18% ID of the labeled mAb L6 detected in the blood 24 h post-injection, compared with about 30% ID for the conjugate. The $t_{1/2}$ for the blood

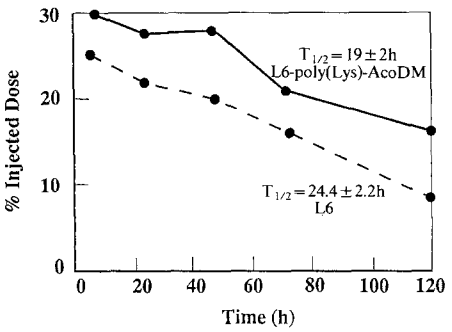

Fig. 8. Plasma clearance of native L6 and its drug conjugate. Most of the details are described in Fig. 7. The percentage of blood in the total mouse (by weight) was considered to be 8%

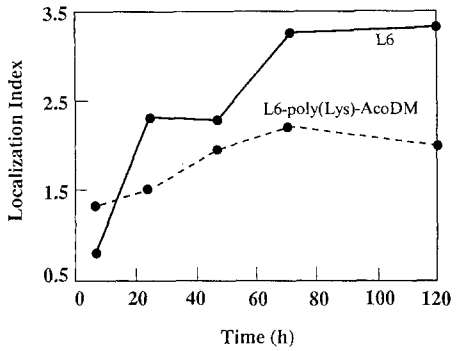

Fig. 9. Change of localization index with time for native L6 and L6-poly(Lys)-AcoDM. The indices were evaluated from Eq. 1, described in Materials and methods; the data for the calculations were obtained from Fig. 7 and 8, and represent an average of counts/organ weight from three animals

clearance of the native mAb and L6-poly(Lys)-AcoDM was 19.0±2 h and 22.8±0.5 h, respectively.

The localization to normal liver and kidney was comparable for the L6-poly(Lys)-AcoDM and mAb L6 (data not shown). The localization index (LI) for mAb L6 and the L6-poly(Lys)-AcoDM was 3.3 and 2.2, respectively (Fig. 9). The LI reached a maximum 72 h after injection for both the conjugate and mAb L6. At the mAb amounts (50 µg mAb total) used for localization studies, no toxic effects were observed following the conjugate injection.

Discussion

The synthesis and pharmacological properties of several DM-mAb conjugates, prepared so that the bond between the drug and the mAb would be acid-cleavable, are described. mAb L6, which was used as the drug carrier, is a non-internalizing antibody (Hellstrom et al. unpublished findings). The aim was to obtain extracellular release of the drug, this type of release being desirable since the drug then can also kill antigen-negative tumor cells.

The measurements of tumor pH values in patients (Table 1), performed as part of this study, confirmed published data. The pH level was approximately 0.8 unit lower in tumor tissue than in normal tissue, [3, 14], and the lower pH in tumors was not dependent on either the type of tumor or its size. Data shown in Fig. 6a, b indicate that approximately 12% of the conjugated drug is released as a result of exposure to the typical tumor pH.

Poly(Lys) was toxic to the tumor cells when tested at high concentrations, as has been observed by others [2, 35]. The molecular-mass dependence of the free poly(Lys) may be related to an increase in the number of positive charges per molecule of the polymer. The decrease in cytotoxicity observed with covalently bound DM was described previously with proteins [32] and polymers [1]. This decrease in activity of the drug can be explained by either the altered transport properties of the linked drug, or by a modification of the drug effect on the cell membrane [7].

The direct linking of DM to the mAb damaged the immunoreactivity and formed unsuitable conjugates, especially at high drug/Ab ratios. However, by inserting a bridge separating the drug from the mAb, it was possible to increase the drug load to an average of 20 molecules/antibody and the mAb binding to its target cells was completely preserved (Fig. 3). There was improved recovery of the conjugate when a step involving precipitation with saturated ammonium sulfate was used, rather than separation on conventional gel-permeation columns: this may have been due to problems caused by absorption of the poly(Lys) or the AcoDM onto the column resins. Purification of the conjugates can be achieved in only a few hours and has been scaled up to provide the amounts needed for in vivo testing. According to analysis on Paragon-gel, the final product contains the conjugate, L6-poly(Lys)-AcoDM, with little or no contamination by free mAb. The overall charge of the antibody seemed to be altered.

The conjugate killed L6-antigen-postitive carcinoma cells in vitro even at neutral pH. The significant increased target cell killing by conjugates at a lower pH (25% and 52% plating efficiency at pH 6 and 7 respectively) was correlated with the increased release of drug from the mAb at a lower pH. The inhibition observed with the melanoma cells may be explained by the volume restrictions imposed by the plate assay. The specificity of killing observed is less than that reportes for some immunotoxins [31] and certain other drug conjugates [6].

The discrepancy between the highly specific binding and the less specific killing may be due to binding of the drug or the polymer to either the target cells or the plastic wells, since such problems have been reported to lead to decreased specificity with adriamycin-T101 conjugate [10]. Localization studies performed in nude mice showed (a) a great similarity between mAb L6 and the L6 poly(Lys)-AcoDM conjugate with respect to uptake in tumor, and (b) that the kinetics of mAb and conjugate were also similar. The clearance and tumor uptake data agreed well with previous reports [20, 26]. Both materials accumulated in tumor to a larger extent than the control mAb 1F5. There was a comparable uptake for the mAb preparations in normal organs such as liver and kidney. The main difference between the conjugate and mAb L6 was a slower clearance of the conjugate from the plasma, which may be explained by its larger size [8]. We cannot predict from the present studies the quantity of the drug that is actually delivered to the tumor. For this purpose labelling of both the antibody and the drug will be needed.

In conclusion, we have prepared an immunoconjugate of mAb L6, which has a pH-labile bond and in which poly(Lys) serves as a spacer between the drug and the mAb. The conjugate has approximately 20 DM molecules/mAb molecule, is stable, binds specifically to tumor cells both in vitro and in vivo and is cytotoxic in vitro to carcinoma cells expressing the L6 antigen particularly after exposure to low pH (range 4–6.5). Further testing will show whether this conjugate is useful for therapy of L6-antigen-positive human tumors growing in nude mice.

The use of agents such as glucose or nigercin [19, 23] is recommended to lower the tumor pH further so as to make our approach practical for clinical use.

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