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Antitumor Activity of L/1C2–4-Desacetylvinblastine-3-carboxhydrazide Immunoconjugate in Xenografts

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ABSTRACT

The murine IgG3 monoclonal antibody L/1C2 is reactive with a high percentage of human carcinomas and has preferentially strong reactivity with tumors of squamous differentiation. This antibody was tested for antitumor activity *in vitro* and in xenograft models as a carbohydrate-linked immunoconjugate with the *Vinca* derivative 4-desacetylvinblastine-3-carboxhydrazide (DAVLBHYD). The conjugate retained good immunoreactivity and was highly active in a cytotoxicity assay. In human tumor nude mouse xenograft studies, L/1C2-DAVLBHYD antitumor activity was superior to that seen with free drug, free antibody, mixtures of free drug and free antibody, or control DAVLBHYD conjugates prepared with non-tumor-binding IgGs. With well-established tumors, potent antitumor activity was observed, including the ability to specifically regress >400-mg tumors to 0 mg. In some cases, apparent long-term cures were effected. In studies using six different human tumor xenografts, the level of potency of L/1C2-DAVLBHYD was related to L/1C2 antigen expression, although the growth rate probably also contributes to the conjugate sensitivity of the tumors.

INTRODUCTION

Targeting of drugs to tumors with monoclonal antibodies continues to show considerable promise for therapeutic utility. This approach offers the potential benefits of favorable drug pharmacokinetics, target selectivity, and, with some antibodies, improved drug internalization. A number of drug and monoclonal antibody combinations have been explored (1–3) among them several utilizing *Vinca* derivatives (4–6). We recently reported (7–9) that a new *Vinca* derivative, DAVLBHYD[2] (2), has significant tumor-suppressive activity when conjugated to the squamous carcinoma-reactive antibodies PF1/D or PF1/B reported by Fernsten *et al.* (10). Although these conjugates represented a step forward, they had two potential shortcomings: (*a*) they showed heterogeneous reactivity with their target tumor, squamous carcinoma; (*b*) although regression of human squamous carcinoma xenografts was demonstrated, the PF1/D-DAVLBHYD and PF1/B-DAVLBHYD conjugates did not consistently completely regress tumors without subsequent regrowth occurring.

Efforts were therefore initiated to generate an additional squamous carcinoma-reactive monoclonal antibody for testing as an immunoconjugate. The antibody selected is designated L/1C2 and is described in detail elsewhere (11). Briefly, L/1C2 is a murine IgG3, reactive with a cell surface, glycoprotein doublet which migrates in the molecular weight range of 110,000–140,000 under reducing conditions. This antigen was shown to internalize after reaction with the L/1C2 antibody, and it is expressed at high density on human squamous carcinomas and on many adenocarcinomas. Normal tissue reactivity includes limited epithelial sites plus endothelium. The present report describes the activity of this antibody as a conjugate with DAVLBHYD in six different human tumor nude mouse xenografts, using several dosing protocols.

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[2] The abbreviations used are: DAVLBHYD, 4-desacetylvinblastine-3-carboxhydrazide; PBS, 0.01 M sodium phosphate, pH 7.4, plus 0.15 M NaCl; LERFI, linear equivalent mean fluorescent intensity.

MATERIALS AND METHODS

Antibody Purification. L/1C2 antibody was produced in serum-free medium (BioResponse, Hayward, CA). Chromatography matrices S-Sepharose Fast Flow, Phenyl-Sepharose Fast Flow, Sephadex G-25, and prepacked columns of Superose 12 for analytical size exclusion fast protein liquid chromatography were purchased from Pharmacia Fine Chemicals.

Monoclonal antibody L/1C2 was purified on a multigram scale using hydrophobic interaction and cation exchange chromatography. Culture concentrate was made 0.5 M in ammonium sulfate by addition of the solid salt. The resultant solution was clarified by centrifugation at 27,000 × *g* for 20 min. The clear supernatant was applied to a column of phenyl-Sepharose Fast Flow previously equilibrated in 100 mM sodium phosphate buffer, containing ammonium sulfate at 0.5 M, pH 7.0 (one l-column bed/15 g antibody). Flow rates for sample application and column development were maintained at 20 cm/h.

Following sample application the column was washed with 1 volume of the phosphate buffer containing 0.3 M ammonium sulfate. The column was then eluted with a linear gradient (5.3 column volumes) of ammonium sulfate, from 0.3 to 0 M, in the phosphate buffer. Fractions were collected during elution and evaluated for immunoglobulin content and purity by size exclusion analysis on Superose 12 columns. Those fractions containing antibody, eluting as a major peak from the phenyl-Sepharose column at an ammonium sulfate concentration of less than 0.12 M, were pooled for further processing.

The pool from the above hydrophobic interaction step was adjusted to pH 5.5 with acetic acid, clarified, and solvent exchanged into 85 mM sodium acetate buffer at pH 5.5 on a Sephadex G-25 column. The solvent-exchanged solution was brought to 25 mM sodium chloride by addition of the solid salt and then chromatographed on a column of S-Sepharose Fast Flow, previously equilibrated in the sodium acetate buffer (one l-column bed/15 g antibody; flow rate, 20 cm/h). Following sample application the column was washed with acetate-buffered 100 mM NaCl. The column was eluted with a linear gradient (1.25-column volumes) of sodium chloride, from 100 to 187 mM, in the acetate buffer, followed by 1.75-column volumes of acetate buffer containing 187 mM sodium chloride. L/1C2-containing fractions eluting at a sodium chloride concentration of 187 mM were pooled and neutralized with the addition of triethanolamine base, sterilized by filtration through 0.2-μm filters, and stored frozen.

Conjugation. DAVLBHYD (12) was generously provided as the hydrogen sulfate salt by G. Cullinan and M. Gleisner, (Lilly Research Laboratories, Indianapolis, IN) and was conjugated to the carbohydrate moiety of the antibody as described (9). Briefly, antibody in 0.1 M sodium acetate buffer (pH 5.6) was treated at 0°C with 160 mM sodium metaperiodate. Excess oxidant was removed by Sephadex G-25 chromatography in the above buffer, and the oxidized antibody was reacted with 5 mM DAVLBHYD at 4°C for 24 h. The resultant L/1C2-DAVLBHYD conjugate was solvent exchanged on Sephadex G-25 into PBS. The conjugation ratio (mol drug/mol antibody) was determined by spectrophotometric methods as described (9), and conjugate concentrations are reported based on the drug (*Vinca*) content of the conjugate. Preparations were sterilized by filtration through 0.2-μm filters and stored at 4°C. Two non-tumor-binding murine IgG1 antibodies, desig-

nated S1 (13) and C-IgG, were conjugated as described above and utilized as negative controls. The latter antibody was kindly provided by J. Starling (Lilly Research Laboratories).

Immunoreactivity and Aggregate Evaluation. An indirect immunofluorescent assay was utilized to evaluate the immunoreactivity of the conjugates. Briefly, target tissue-cultured cells were removed from tissue culture flasks with trypsin/EDTA (Gibco). Cells (1×10^6) were washed and reacted with antibody preparations for 1 h at 4°C. The cells were washed free of unreacted antibody with a fetal calf serum underlay and washed again with Hanks' balances salt solution (Gibco). Fluorescein isothiocyanate-labeled goat $F(ab')_2$ anti-mouse IgG was added, and the cells were incubated and washed as indicated above. They were finally resuspended in 1% formaldehyde in PBS and evaluated for fluorescence with an EPICS-Coulter Mark IV cell analyzer. Fluorescence intensity was expressed as LERFI. LERFI is computed by converting fluorescent intensity collected by log amplifiers to linear units (14). This allows a more relevant direct comparison of fluorescent values.

Aggregate formation was evaluated using a Superose 12 HR10/30 gel filtration column (Pharmacia) and a Pharmacia fast protein liquid chromatography system (15). Samples were applied in 0.3 ml 0.1 M Trizma (Sigma Chem Co., St Louis, MO) buffer, pH 8.0, plus 0.1 M NaCl and eluted in the same buffer at 0.5 ml/min.

Cell Lines and Xenografts. The human carcinoma cell lines FADU (ATCC HTB43), MEL 80 (ATCC HTB33), HT29 (ATCC HTB38), LS174T (ATCC CL188), and UCLA/P3 (16) were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. T222 (17) cells were grown in similarly supplemented RPMI 1640 medium. Prior to each xenograft experiment, cells were collected by treatment with trypsin/EDTA (Gibco, Life Technologies, Grand Island, NY) and washed with supplemented Dulbecco's minimal essential medium and finally suspended in Hanks' balanced salt solution. Cells (1×10^7) were injected s.c. into the hips of young adult female nude mice (Charles River Breeding Laboratories, Boston, MA). The mice were treated by i.v. injection in the tail vein at various times. Tumor measurements were taken in two dimensions and converted to an estimate of mass using the formula [(length) (width^2)/2] as described (18). Control groups contained 10 mice, with test groups containing 5 mice each. The Student's *t* test was used to evaluate differences between mean tumor masses.

Membrane Preparation. Membranes for absorption studies were prepared from approximately 2-week-old tumor xenografts using the methods described by Howard *et al.* (19). Absorptions were carried out by allowing 50 μl antibody at 5 μg/ml to react with 20 μg membrane preparation for 30 min at melting ice temperatures. The supernatant was then reacted with target T222 cells using the indirect immunofluorescent assay described above. Student's *t* test was used to evaluate differences in antigen expression.

RESULTS AND DISCUSSION

The studies described here required significant quantities of antibody. Efforts were made therefore to develop the large scale purification protocols detailed in "Materials and Methods." This procedure resulted in an overall yield in excess of 70%. Purity, as estimated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, was greater than 95%, with undetectable levels of aggregate as determined by Superose 12 chromatography.

L/1C2 typically conjugated to DAVLBHYD with conjugation ratios (mol drug/mol protein) of 4–7. Conjugates had good solubility characteristics, remaining in solution at 4°C in PBS at concentrations in excess of 10 mg/ml. As is indicated in Fig. 1, immunoreactivity with the antigen-positive cell lines T222 and FADU was retained postconjugation (Fig. 1, Panels 2 and 16), in comparison to the reactivity of unmodified antibody (Fig. 1, Panels 1 and 15). Results from using antibody concentrations of 0.5 μg/ml are shown, and the actual linearized mean fluorescent values are given in the legend to Fig. 1. Although

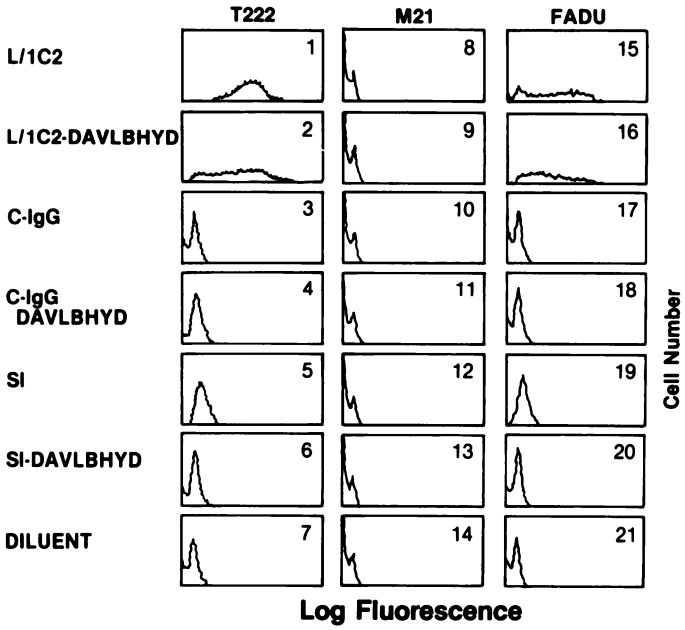

Fig. 1. Fluorescence-activated cell sorter analyses of reactivity of antibodies and antibody-drug conjugates with human tumor cells. All preparations were tested at 0.5 μg/ml. Target cells included the T222 (*1–7*) and FADU (*15–21*), both of squamous carcinoma origin, and the melanoma line M21 (*8–14*).

Reactivity of the indicated antibodies and antibody-*Vinca* conjugates was determined using an indirect technique using fluorescein isothiocyanate-labeled goat F(ab')2 anti-mouse IgG. C-IgG and S1 are both IgG1 monoclonal antibodies which do not bind to squamous carcinoma targets and are used in subsequent experiments as control drug conjugates. Linearized mean fluorescence values for each panel are as follows: *1*, 36.9; *2*, 27.9; *3*, 1.8; *4*, 2.2; *5*, 3.1; *6*, 2.0; *7*, 1.8; *8*, 1.5; *9*, 1.6; *10*, 1.5; *11*, 1.6; *12*, 1.5; *13*, 1.5; *14*, 1.6; *15*, 22.4; *16*, 19.3; *17*, 1.6; *18*, 1.9; *19*, 2.6; *20*, 1.9; 21, 1.8.

the reactivity profiles were somewhat altered postconjugation, linearized mean fluorescent values of the conjugate relative to the unmodified antibody values indicated a reduction in reactivity of 24 and 14% with T222 and FADU targets, respectively. When the linearized mean fluorescent values were compared for L/1C2-DAVLBHYD and free L/1C2 at the higher concentration of 5 μg/ml, similar results were obtained (L/1C2-DAVLBHYD: T222, 92.9; FADU, 47.7; L/1C2: T222, 71.0; FADU, 36.2) This translated to a reduction postconjugation at 5 μg/ml of 23.6 and 24.1% for T222 and FADU targets, respectively. The antigen-negative cell line M21 was unreactive as expected.

Irrelevant antibodies C-IgG and S1 (both IgG1) conjugated to DAVLBHYD in much the same manner as L/1C2 (IgG3), yielding products with similar conjugation ratios and aggregate content. As is also indicated in Fig. 1, these antibodies and corresponding conjugates did not bind significantly to the tumor cell targets used in these studies. It should be noted that, as has been discussed previously (7, 8), the non-tumor-binding conjugates were used to control for nonspecifically altered *in vivo* drug pharmacokinetics. They were selected primarily based on their biochemical profiles (conjugation ratio, aggregate content, solubility) rather than their isotype. IgG3 control conjugates with these vital characteristics have not yet been identified.

L/1C2-DAVLBHYD and controls were tested for antitumor activity in several human tumor nude mouse xenograft models. Fig. 2 summarizes data from an experiment in which T222 human squamous carcinoma cells were grown as xenografts on the hips of nude mice. When the tumors had grown for 7 days, and averaged 126 ± 12 (SE) mg, groups of mice were given a single i.v. injection of the L/1C2-DAVLBHYD, irrelevant control conjugate, or control compounds as indicated. All groups

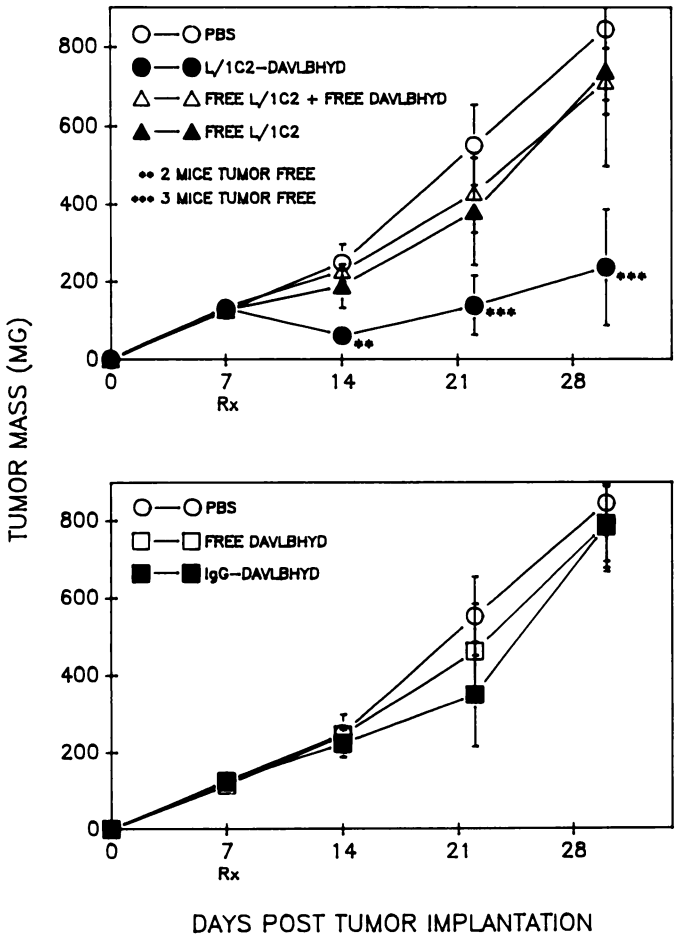

Fig. 2. Efficacy of L/1C2-DAVLBHYD conjugate and controls in T222 squamous carcinoma xenografts. T222 tumor cells were injected s.c. in the hips of nude mice and allowed to grow for 7 days prior to therapy. The mice were then given a single injection of the indicated compounds at 2 mg *Vinca*/kg or 75 mg protein/kg, the latter being the protein content of a conjugate dose containing 2 mg *Vinca*/kg. Tumor masses ± SE (*bars*) are indicated. The PBS-treated control group contained 10 mice and experimental groups contained 5 mice each. The data shown represent a single experiment separated into two graphs for clarity. The plot of the PBS-treated control group shown on the *top panel* is repeated on the *bottom panel* for purposes of comparison. The IgG-DAVLBHYD control conjugate in this experiment was made with the non-tumor-binding antibody S1.

received 2 mg/kg (*Vinca* content) or the protein equivalent of that dose (75 mg protein/kg). Control treatments included free L/1C2, free DAVLBHYD, a mixture of free L/1C2 plus free DAVLBHYD, and the non-tumor-reactive IgG conjugate. None of the controls had significant antitumor activity when compared to each other or to a group of mice injected with PBS. Only the group treated with L/1C2-DAVLBHYD exhibited significant tumor regression, with 2 of 5 mice being tumor free by day 14 and 3 of 5 being tumor free on days 22 and 30, when the experiment was terminated.

Fig. 3, *A–C* shows an experiment in which T222 squamous carcinomas were allowed to grow to an average size of 421 ± 96 mg. The mice were then treated by i.v. injection on a 6-dose protocol over a 3-week period at the indicated doses. Neither non-tumor-binding control conjugate nor free DAVLBHYD had antitumor activity relative to a PBS-treated group. In contrast, L/1C2-DAVLBHYD treatment at 1 mg/kg (*Vinca* content)/injection resulted in complete regression of tumors in 5 of 5 mice. These mice remained tumor free throughout the experiment. The tumor-free mice were retained and showed no sign of tumor regrowth beyond 165 days. L/1C2-DAVLBHYD dosed at 0.5 mg/kg (*Vinca* content)/injection also induced tumor regression, although at this lower dose there was subsequent tumor regrowth in 2 of 5 mice. L/1C2-DAVLBHYD dosed at 0.25 mg/kg (*Vinca* content) had no significant antitumor activity in this model.

Fig. 3, *D–F*, depicts a parallel therapy experiment carried out using nude mice bearing FADU squamous carcinoma xenografts with an average size of 418 ± 84 mg. Again, a 6-dose protocol was used. In this model, free DAVLBHYD and non-tumor-binding control conjugate showed dose-dependent antitumor activity, with significant regressions occurring in the groups treated at the 1 mg/kg (*Vinca* content) level. Reduced antitumor effects were seen with the lower doses. In contrast, L/1C2-DAVLBHYD at all three dose levels [1, 0.5, and 0.25 mg/kg (*Vinca* content)] completely regressed all tumors in all groups. The tumor-free conjugate-treated mice remained tumor free beyond 165 days. It is noteworthy that, throughout the above studies, the antitumor activity of the L/1C2-DAVLBHYD conjugate was consistently superior to controls including free-drug and irrelevant conjugate.

The above studies led to the conclusion that L/1C2-DAVLBHYD could kill tumor cell targets more effectively than free drug or irrelevant conjugate. We next explored the relationships among level of antigen expression, growth rate, and conjugate sensitivity, using a variety of human tumor xenograft targets. In all cases identical dosing protocols were used, mice being injected in the tail vein on days 3, 6, and 9 after implantation of 1×10^7 tumor cells s.c. in the flanks of the mice. With dose-ranging studies, a minimal dose needed to induce greater than 50% tumor suppression relative to diluent-treated control mice was estimated. Table 1 summarizes these studies. Antigen expression of exponentially growing target tumor cell lines is indicated as a linearized fluorescence value (see "Materials and Methods"). Also indicated is the estimated tumor mass of the control mice at 21 days after tumor implantation as a reflection of growth rate.

These data suggest that antigen expression can influence the sensitivity of a given target cell to L/1C2-DAVLBHYD. For example, T222 is more sensitive to the conjugate activity (minimal effective dose, 0.15 mg/kg) than HT29 (minimal effective dose, 0.5 mg/kg); these two cell lines had statistically indistinguishable growth at 21 days (703 ± 55 and 638 ± 62 mg, respectively; $P > 0.1$) with higher antigen expression found on T222 ($P < 0.001$).

In seeming contrast, UCLA/P3 and LS174T were equally sensitive to the conjugate, although fluorescence on the LS174T cell line was lower ($P < 0.001$). The explanation for this result appears to lie in the *in vivo* expression of the L/1C2 antigen. When the L/1C2 antibody was absorbed with a membrane preparation from an LS174T xenograft, levels of absorption (and hence antigen expression) were found to be similar to that observed with T222 membrane preparations (Table 2). This suggests that LS174T expresses higher levels of L/1C2 antigen *in vivo* than *in vitro*. Indeed, the apparent high level of L/1C2 antigen expression in the LS174T tumor *in vivo* would suggest that this tumor might have been more sensitive to L/1C2-DAVLBHYD conjugate that it was. The explanation for this may lie in the aggressive growth of this tumor, which was more than twice the size of the T222 tumor in 21 days (LS174T: 2006 ± 251 mg; T222: 703 ± 55 mg; $P < 0.001$)

Membrane preparations from the remaining cell lines were also tested for their ability to absorb L/1C2 antibody. When ranked based on this activity, they fell into the same rank order as was observed when they were ranked based on *in vitro* fluorescence (Table 1), indicating a good correlation between

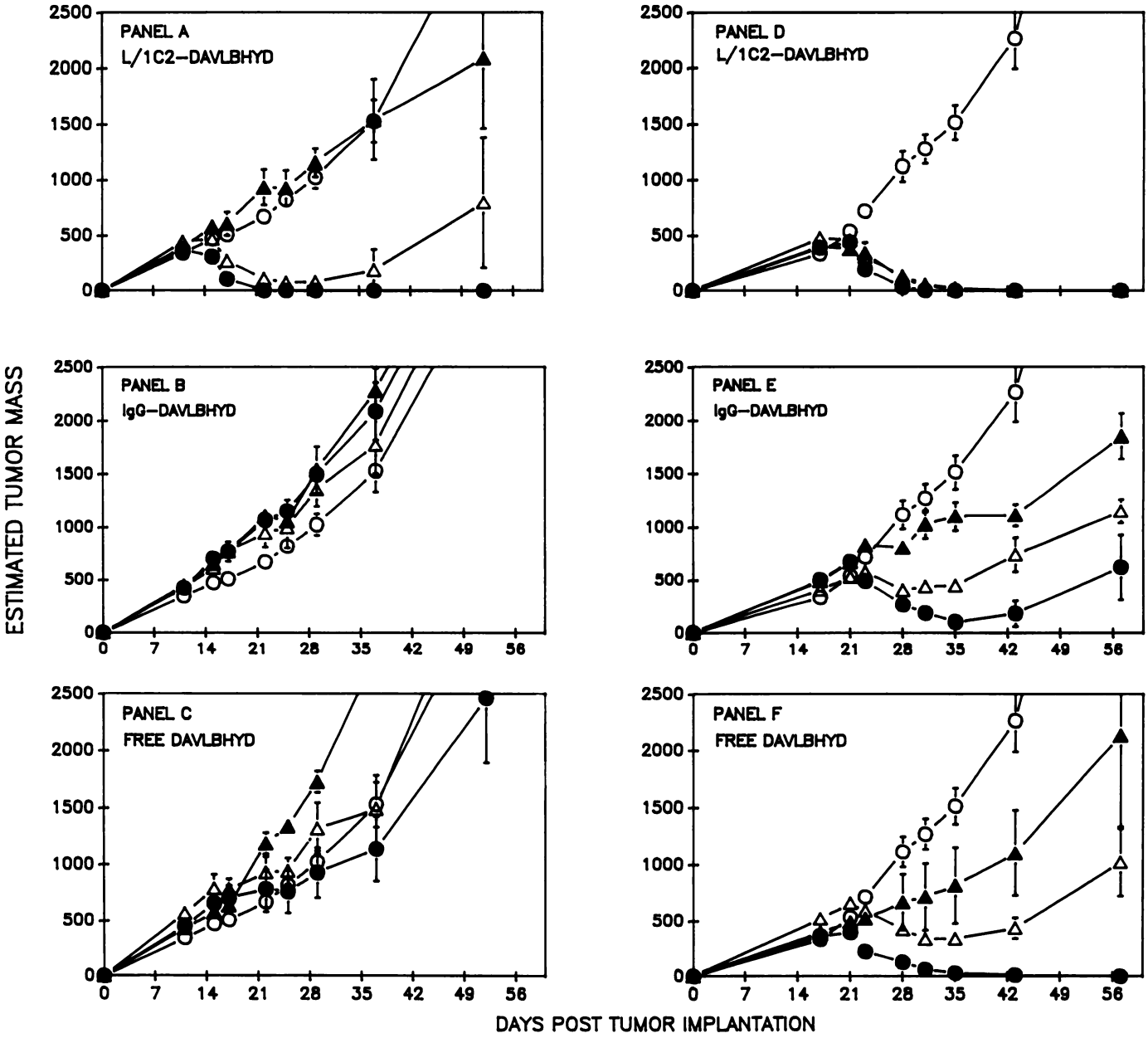

Fig. 3. Efficacy of L/1C2-DAVLBHYD conjugate and controls in T222 (*A*, *B*, and *C*) and FADU (*D*, *E*, and *F*) squamous carcinoma xenografts. Tumor cells were injected s.c. in the flanks of nude mice and allowed to grow to 400–450 mg in size prior to therapy. Therapy consisted of 6 i.v. doses in the tail vein given on days 11, 15, 17, 22, 25, and 29 at the doses indicated below. Free antibody doses were equivalent to the protein content of the corresponding conjugate dose. Tumor masses ± SE (*bars*) are indicated. The PBS-treated control group contained 10 mice and experimental groups contained 5 mice each. The IgG-DAVLBHYD control conjugate in this experiment was made with the non-tumor-binding antibody C-IgG. ○, PBS control; ●, 1 mg/kg; △, 0.5 mg/kg; ▲, 0.25 mg/kg.

Table 1 *L/1C2 antigen expression, potency, and growth*

Target	LERFI[a]	Minimal effective dose[b] (mg *vinca*/kg)	Tumor mass at 21 days (±SE)[c]
T222 (Sq[d])	14.7 ± 1.4	0.15	703 ± 55
FADU (Sq)	16.0 ± 1.9	0.12	473 ± 46
ME180 (Sq)	12.3 ± 0.6	0.25	318 ± 49
UCLA/P3 (Adeno)	10.6 ± 0.2	0.25	2311 ± 471
LS174T (Adeno)	7.8 ± 0.5	0.25	2006 ± 251
HT29 (Adeno)	5.5 ± 0.4	0.50	638 ± 62

[a] See "Materials and Methods" and Ref. 14.
[b] Minimal conjugate dose expressed as drug content needed to achieve 50% tumor suppression 21 days after tumor implantation in mice treated by i.v. injection on days 3, 6, and 9.
[c] Estimated tumor mass of PBS control groups on day 21 after tumor implantation. Ten mice per group.
[d] Sq, squamous carcinoma origin; Adeno, adenocarcinoma origin.

Table 2 *Xenograft membrane absorption of L/1C2 antibody*

Fifty μl of L/1C2 antibody at 5 μg/ml was incubated with the indicated membrane preparations (20 μg) for 30 min on ice. The supernatant was then reacted with T222 target cells in an indirect fluorescence assay. LERFI (Ref. 14) is indicated, with reduced fluorescence relating to absorption of antibody activity by the membrane preparations.

Membrane source	LERFI *vs.* T222 target
Unabsorbed	12.55 ± 0.78
LS174T (Adeno[a])	4.65 ± 0.07
T222 (Sq)	5.80 ± 0.14
FADU (Sq)	10.50 ± 0.14
ME180 (Sq)	12.15 ± 0.78
UCLA/P3 (Adeno)	12.40 ± 0.28
HT29 (Adeno)	13.10 ± 0.28

[a] Sq, squamous carcinoma origin; Adeno, adenocarcinoma origin.

antigen expression *in vitro* and *in vivo* with these cell lines. In the absorption assay, however, only minimal activity was seen with the ME180, UCLA/P3, and HT29 membranes, suggesting

that the levels of antigen present in those preparations was near the sensitivity limit for this assay.

In summary, these studies describe an antibody drug conjugate with potent antitumor activity which is superior to that obtainable with the corresponding free drug, free antibody, or irrelevant control conjugates. The conjugate sensitivity of target cell lines was generally correlated with antigen expression, although growth rate may also play a role in determining conjugate sensitivity of a tumor. Determining the future of the L/1C2-DAVLBHYD conjugate as a therapeutic candidate for human squamous carcinoma will be dependent on careful evaluation of the significance of the normal tissue reactivity of this antibody. With regard to the latter, our recent studies suggest that the L/1C2 antibody may be weakly reactive with the H type 2 carbohydrate antigen.[3] This finding is consistent with the reported endothelial reactivity of the antibody (11). *In vivo* toxicology studies in antigen-positive nonhuman primates have been initiated to explore the significance of this reactivity.

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[3] Unpublished observation.