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In vivo efficacy of monoclonal antibody – drug conjugates of three different subisotypes which bind the human tumor – associated antigen defined by the KS1/4 monoclonal antibody

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Summary. A panel of three hybridomas has been isolated each of which secretes a single species of monoclonal antibody (MoAb) directed against the KS1/4 tumor-associated antigen originally described by Varki et al. (*Cancer Res* 44: 681, 1984). These MoAbs were designated L1-(IgG_{2b}), L2-(IgG_1), and L4-(IgG_{2a}) KS. Binding specificity, immunoprecipitation, and competitive binding analyses indicated that these MoAbs each recognize the same epitope of the KS1/4 antigen. The immunoprecipitation studies indicated that the MoAbs recognized a major antigenic component of 42 kDa and a minor component of 35 kDa. The L-KS antibodies were evaluated as MoAb-drug conjugates against a variety of human tumor targets grown in vivo as nude mouse xenografts. The MoAb-drug conjugates were constructed using protein-A-purified MoAbs conjugated to 4-desacetyl-vinblastine-3-carboxhydrazide. Efficacy was determined using various dosing protocols on 2–14 day established tumors of lung, pharynx, colon, and skin origin. Control experiments included the use of dual-flank antigen-positive and negative tumors, free MoAbs, free drug, and mixtures of MoAbs and drug. These studies indicated that significant tumor growth supression and actual tumor regression could be achieved by the MoAb – vinca conjugates and that this activity was antigen-mediated. The drug conjugates were more efficacious than free drug or free MoAbs administered either singly or in combination with each other.

Introduction

The lymphocyte hybridoma technique discovered by Kohler and Milstein [13] has enabled the isolation of monoclonal antibodies (MoAbs) directed against a vast array of antigenic targets. Many laboratories have taken advantage of hybridoma technology to produce MoAbs which bind to tumor-associated antigens from a large number of tumor types [18, 23, 29, 32]. MoAbs directed against human tumor-associated antigens may be utilized in the diagnosis and therapy of human tumors [21]. Several reports have already emerged describing the in vivo therapeutic activity of murine MoAbs in patients with lymphoma [15], colorectal carcinoma [22], and melanoma [10, 20]. The potential therapeutic utility of tumor-binding MoAbs that are coupled to a variety of cytotoxic agents, such as drugs, radioisotopes, and toxins, is an area of intense research interest in a number of laboratories [2, 5, 17]. Initial clinical results, utilizing an immunotoxin directed against melanoma and a MoAb – neocarzinostatin conjugate reactive with colorectal carcinoma, have recently been reported [24, 28].

Varki et al. [30] described the isolation of a MoAb, KS1/4, which was reactive with a 40-kDa glycoprotein found on the cell surface of UCLA-P3 human lung adenocarcinoma cells used to activate mouse B lymphocytes for hybridoma production. This MoAb exhibited a broad reactivity to a number of tumor types including adeno-, epidermoid, and small-cell carcinoma of the lung; as well as stomach, breast, and pancreatic carcinomas [30]. The KS1/4 determinant may be an oncofetal antigen because the molecule was found on fetal lung, kidney, and colon [30]. The KS1/4 MoAb has been chemically conjugated to vinca alkaloids [12] and methotrexate [31] and has demonstrated potent tumor suppressive activity against UCLA-P3 tumors grown in nude mice [12, 31]. In order to assess more fully the potential of the KS1/4 antigen to serve as a target for MoAb-drug-conjugate-mediated cytotoxicity, our laboratory has isolated a panel of three MoAbs of different isotypes each binding to the same epitope of the KS1/4 antigen. These antibodies offered an unique opportunity to extend previous immunoconjugate studies using this antigen system and to examine the effect of the antibody subisotype on the action of MoAb-drug conjugates.

Materials and methods

Cell lines. All human tumor cell lines were purchased from the American Type Culture Collection (Rockville, Md) except for UCLA-P3 lung adenocarcinoma [30] and M14 and M21 melanoma [4] cells, which were kindly provided by Dr. Thomas Bumol, Lilly Research Laboratories, Indianapolis, Ind. The cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco laboratories, Gaithersburg, Md) containing 10% fetal calf serum (Hyclone laboratories, Logan, Utah) and 50 μg/ml gentamicin (Gibco).

Immunizations. UCLA-P3 lung adenocarcinoma cells were grown to 70–80% confluency in tissue culture and removed from the substratum with Ca^{+2}- and Mg^{+2}-free phos-

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phate-buffered saline (PBS) containing EDTA and glucose [19]. The cells were washed three times in PBS and $(0.3-2)\times 10^7$ intact cells in a volume of 0.1–0.2 ml were injected at weekly intervals into Balb/c (Harlan Sprague Dawley, Indianapolis, Ind) or Robertsonian [27] mice (Jackson Laboratories, Bar Harbor, Me). The Balb/c mice received four intraperitoneal (i.p.) injections while the Robertsonian mice were injected three times i.p. and once intravenously.

Fusion. Four days after the last immunization the spleens were removed from the mice and activated lymphocytes were fused to HL-1 Friendly-653 (F.653) nonsecreting myeloma cells (Ventrex Laboratories, Portland, Me) according to standard hybridoma methods [7]. Briefly, the spleens were removed and dispersed into single cells by forcing the tissue through a stainless-steel mesh. Red blood cells were lysed with 0.17 M NH_4Cl and the resulting B lymphocytes were washed in serum-free medium. Splenocytes (1.5×10^7 cells) were fused with 3×10^7 F.653 myeloma cells in the presence of 42% poly(ethyleneglycol) (American Type Culture Collection) and 6% dimethylsulfoxide (Sigma Chemical Co., St. Louis, Miss). The cells were centrifuged and resuspended in 60 ml Dulbecco's modified Eagle's medium containing 20% fetal calf serum with 1×10^{-5} M hypoxanthine, 1×10^{-6} M aminopterin, 3×10^{-6} M thymidine (HAT, Balb/c splenocytes) or 7.5×10^{-5} M adenine, 1×10^{-6} M aminopterin, 3×10^{-6} M thymidine (AAT, Robertsonian splenocytes) and mixed with 1–2 ml macrophages obtained by peritoneal washout of 1 Balb/c or Robertsonian mice (hypoxanthine, adenine, aminopterin, and thymidine were purchased from Sigma). The cells were dispersed in 0.2-ml aliquots in 96-well microtiter plates.

Hybridoma screening. Seven to ten days after the initial seeding, actively growing hybridomas were screened for antibody production to human cell lines and membrane fractions obtained from autopsy specimens using a solid-phase radioimmunoassay (RIA) according to methods previously described [25]. Antigen localization to the membrane was accomplished using indirect immunofluorescence against live UCLA-P3 cells. This was accomplished by removing the cells from the tissue-culture substratum with EDTA treatment and washing the cells in cold tissue-culture medium. Aliquots of 1×10^6 cells were placed in tubes and 50 µl hybridoma supernatant containing 0.02% azide were added to the cells and incubated for 45 min at 4° C. The cells were washed in cold medium and 50 µl 1:100 dilution of rabbit anti-(mouse IgG)–fluorescein-isothiocyanate (Jackson Immuno Research Laboratories, Avondale, Pa) containing 0.02% azide were added and incubated for an additional 45 min. The cells were washed and examined for fluorescence under a UV microscope or analyzed with an EPICS/Coulter Mark IV cell analyzer. Mean fluorescence intensities, obtained from flow cytometry experiments, were converted to linear equivalent units by multiplying them by 0.0118 and taking the antilog. Control myeloma immunoglobulins were purchased from Litton Bionetics (Kensington, Md).

Competitive binding inhibition. The ability of hybridoma supernatants to inhibit competitively the binding of ^{125}I-labeled KS1/4 MoAb (protein-A-purified KS1/4 provided by Dr. Thomas Bumol, Lilly Research Laboratories) was determined by incubating 7.5 µg/ml protein-A-purified MoAbs with glutaraldehyde-fixed UCLA-P3 cells for 1 h. The cells were washed and 200000 cpm ^{125}I-KS1/4 (labeled by the iodobead method [16] were added for 1 h. The cells were washed again and counted in a gamma counter.

Immunoprecipitation. Immunoprecipitation analyses were done by reacting detergent-soluble extracts of [^{3}H]-glucosamine-labeled UCLA-P3 cells with protein-A – Sepharose – MoAb complexes [3]. Briefly, four T-150 flasks of UCLA-P3 cells were grown for 48 h in the presence of 10 µCi/ml D-[1,6-^{3}H]glucosamine hydrochloride (New England Nuclear, Boston, Mass). The cells were removed from the substratum with EDTA and washed in PBS. The resulting cell pellet was solubilized in RIPA lysis buffer [3] and the detergent-solubilized material was clarified by a 15-min centrifugation in a Beckmann (Palo Alto, Calif) Airfuge at 207 kPq (30 lb/in^2). The labeled antigen preparation was incubated with protein-A–MoAb complexes prepared as described [3]. The MoAb-absorbed antigens were solubilized in Laemmli [14] buffer with 1% mercaptoethanol, heated at 100° C for 5 min, and subjected to SDS/polyacrylamide gel electrophoresis. The gel was treated with Enlightening (New England Nuclear), dried down, and autoradiographed in Dupont (Wilmington, Del) high speed X-ray cassettes using Kodak X-Omat AR film (Rochester, NY).

MoAb-drug conjugation and characterization. Ascites – or in-vitro-produced MoAbs, obtained from doubly cloned hybridoma cells, were protein-A-purified and conjugated to 4-desacetylvinblastine-3-carboxhydrazide (DAVHYD) [11]. Briefly, MoAbs were concentrated to about 10 mg/ml by vacuum dialysis in PBS and subsequently dialyzed against 0.1 M sodium acetate pH 5.6. MoAbs were oxidized by treatment with 160 mM sodium metaperiodate and purified by Sephadex G-25 chromatography in the acetate buffer. Conjugation to DAVHYD was performed by incubating the MoAb for 24 h at 4° C in the presence of 5 mM vinca. The resulting MoAb-drug conjugates were purified by Sephadex G-25 in PBS. This procedure routinely resulted in the conjugation of 4–6 mol drug/mol MoAb. Retention of antigen-binding capacity was determined by solid-phase RIA against both antigen-positive and -negative cell targets. Aggregate formation was evaluated using a Superose 12 HR 10/30 gel filtration column (Pharmacia, Piscataway, NJ) and a Pharmacia fast-protein liquid chromatography system [1]. Samples were applied in 0.1 M Tris (Sigma) buffer, pH 8, plus 0.1 M NaCl and eluted in the same buffer.

Nude mouse xenografts. The human tumor cell lines were grown in vitro and 1×10^7 cells were injected subcutaneously into the rear flanks of 20–25-g female outbred nude mice (Charles River Breeding Laboratories, Boston, Mass). The tumors were allowed to establish for various times and treatments of free drug, free MoAb, mixtures of drug and MoAbs, or MoAb-DAVHYD conjugates were given intravenously at defined intervals. Tumor size was determined by caliper measurements and tumor mass in milligrams was estimated from the formula $l\times w^2/2$ where l is the length and w is the width of the tumor mass in millimeters [6]. All control groups contained ten animals whereas the treatment groups comprised five mice each.

Table 1. Comparison of L-KS MoAb immunological reactivity with KS1/4[a]

Target[c]	MoAb[b]			
	KS1/4	L1-KS	L2-KS	L4-KS
Tumor				
UCLA-P3	27900	21600	15800	14300
CaLu-1	3600	5100	15200	13700
CaCo-2	28400	20100	9400	10200
DU145	19400	16700	7000	6000
PC-3	7100	6700	4500	4300
T-24	1000	1100	NT	NT
Normal				
IMR-90	0	NT	300	300
PBL	0	0	300	500
Colon	9000	7500	5800	7600
Liver	1100	NT	1000	200
Prostate	1600	800	800	0
Kidney	1600	2800	3400	1900

[a] Reactivity determined using cells and tissues in a solid-phase radioimmunoassay [25]. Data expressed as test MoAb (cpm) minus control myeloma IgG2a (cpm)
[b] MoAbs were in tissue-culture supernatants. KS1/4 concentration was 30 μg/ml. L-KS concentrations were 5–20 μg/ml
[c] UCLA-P3 and CaLu-1, human lung tumor cell lines; CaCo-2, colorectal tumor line; PC-3 and DU145, prostate tumor lines; T-24, bladder cell line; IMR-90, normal human fibroblast line; PBL, peripheral blood lymphocytes, the remaining targets are derived from membrane preparations of human surgical or autopsy specimens [25]
NT, Not tested

Results and discussion

The screening strategy employed to identify new hybridomas secreting MoAbs directed against the KS1/4 antigen involved a multi-tiered approach. Culture supernatants were first screened against UCLA-P3 lung adenocarcinoma and M-21 melanoma cells in a solid-phase RIA. Antibodies reactive with UCLA-P3 and negative against M-21 were screened against hematopoietic cells. MoAbs not reactive to blood cells were tested by live-cell immunofluorescence against UCLA-P3 tumor cells. Membrane-reactive hybridoma supernatants were then screened against an expanded panel of cell lines and tissues in a solid-phase RIA. MoAbs which exhibited a similar reactivity pattern as KS1/4 were then examined in competitive binding and immunoprecipitation assays. A total of 1123 hybridomas (163 from Balb/c and 960 using Robertsonian lymphocytes) were screened in this way. Three new hybridoma clones, designated L1-, L2-, L4-KS, were identified, which secreted MoAbs that bound to a variety of cells and membrane extracts in a pattern similar to KS1/4 (Table 1). The L1-KS hybridoma was derived from Balb/c splenocytes while L2- and L4-KS utilized Robertsonian lymphocytes. The data in Table 1 indicate that while quantitative differences in binding were noted between the L-KS MoAbs and KS1/4, the qualitative aspects of MoAb reactivity were quite similar. The quantitative differences may have been due to variable levels of IgG in the culture supernatants, MoAb affinity, or preferential binding by the iodinated secondary antibody to a particular MoAb subisotype. Competitive binding inhibition studies (Table 2) indicated that the L-KS MoAbs were potent inhibitors of ^{125}I-KS1/4 binding to target UCLA-P3 tumor cells. F12-1C11 is an IgG_{2b} MoAb which binds to a membrane antigen on UCLA-P3 tumor cells and which is distinct from KS1/4 (data not shown). This MoAb was not effec-

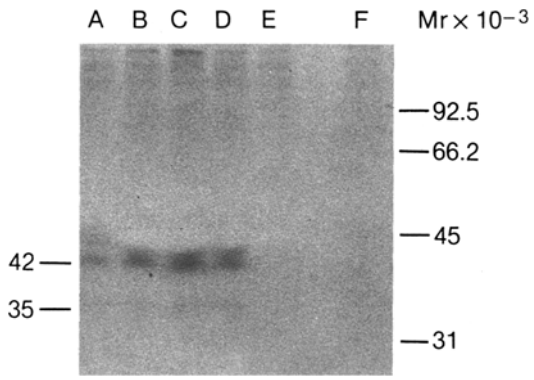

Fig. 1. Immunoprecipitation of [^{3}H]glucosamine – labeled detergent extracts of UCLA-P3 human lung adenocarcinoma cells by L-KS and KS1/4 MoAbs. UCLA-P3 tumor cells were metabolically labeled with [^{3}H]glucosamine and solubilized in RIPA lysis buffer [3]. The detergent extracts were incubated with protein-A–Sepharose–rabbit-anti-(mouse immunoglobulin)–murine-MoAb complexes [3]. The MoAb affinity matrices were washed and heated at 100° C for 5 min in Laemmli buffer [14] containing 1% mercaptoethanol. Eluted fractions were subjected to Laemmli [14] discontinuous SDS/polyacrylamide gel electrophoresis using a 12% separating gel and 4% stacking gel. The gel was stained with Coomassie blue, destained, treated with Enlightning (New England Nuclear), dried down, and subjected to fluorography. Samples were loaded with respect to maximum radioactivity as follows: *A*, 8000 cpm L1-KS; *B*, 8000 cpm L2-KS; *C*, 8000 cpm L4-KS; *D*, 8000 cpm KS1/4; *E*, 4800 cpm murine myeloma IgG_1 control; *F*, 3700 cpm murine myeloma IgG_{2b} control. The X-ray film was developed at −70° C in a high-speed cassette (Cronex, DuPont) for 43 days. Samples were overloaded with respect to protein, which caused some anomoly in the migration of the L1-KS specimen (*lane A*). Other experiments have indicated that the L1-KS MoAb immunoprecipitation profile is indistinguishable from the other L-KS MoAbs as well as KS1/4 (data not shown)

Table 2. Competitive binding inhibition of ^{125}I-KS1/4 MoAb to UCLA-P3 human lung adenocarcinoma cells L-KS MoAbs[a]

Blocking MoAb[b]	^{125}I-KS1/4 bound to UCLA-P3 (cpm)	Inhibition[c] (%)
None	15000	0
KS1/4	6700	55
L1-KS	2900	81
L2-KS	3600	76
L4-KS	3400	77
F12-1C11[d]	13000	13

[a] Assay performed by incubating 7.5 μg/ml blocking MoAbs with glutaraldehyde-fixed UCLA-P3 for 1 h at room temperature. Cells were washed and 200000 cpm ^{125}I-KS1/4 were added for 1 h. The cells were washed again and counted in a gamma counter
[b] MoAbs were purified by protein A
[c] Inhibition (%) = $[1-(a/b)]\times 100$; where a = ^{125}I-KS1/4 bound (cpm) in presence of test MoAb and b = ^{125}I-KS1/4 bound (cpm) in absence of test MoAb
[d] F12-1C11 is an IgG_{2b} MoAb which binds to UCLA-P3 tumor cells but is not reactive with the KS1/4 antigen

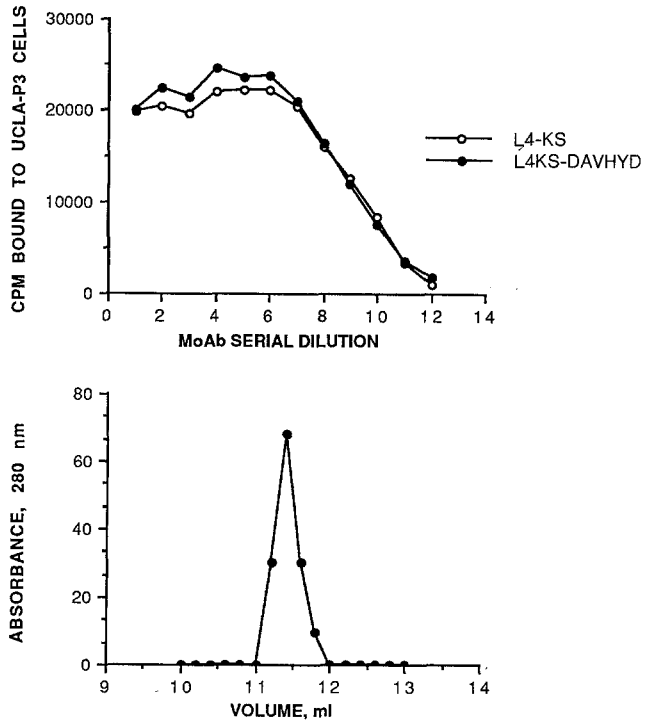

Fig. 2. Antigen-binding activity and aggregate content of L4-KS–DAVHYD. *Top panel,* the antigen-binding activity of unmodified L4-KS MoAb and L4-KS–DAVHYD against UCLA-P3 human lung tumor cells as determined by a solid-phase radioimmunoassay (RIA) [25]. The MoAb or MoAb-drug conjugate stock concentration was 30 μg/ml. *Bottom panel,* the Superose 12 elution profile of the L4-KS–DAVHYD conjugate in the solid-phase RIA. Absorbance at 280 nm is reported on a relative scale of 1–100. The L4-KS–DAVHYD conjugate had a conjugation ratio of 4.4 molecules of drug per molecule of IgG

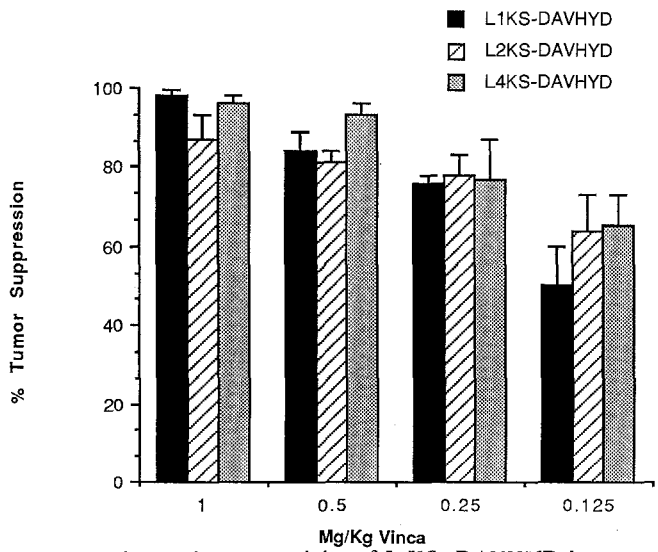

Fig. 3. In vivo anti-tumor activity of L-KS–DAVHYD immunoconjugates. Nude mice were injected subcutaneously with 1×10^7 UCLA-P3 tumor cells. MoAb-drug conjugates were given intravenously at days 2, 4, and 7 after tumor implantation. The abscissa is the conjugate dose given in mg/kg vinca equivalent while the ordinate is tumor suppression (%). Tumor suppression is defined as $[1-(a/b)] \times 100$ where a = tumor mass of the treatment animal and b = mean tumor mass of the saline-injected control group. The data shown were obtained 28 days after tumor implantation. Standard error bars are included

tive at inhibiting ^{125}I-KS1/4 binding (Table 2). Immunoprecipitation analysis (Fig. 1) demonstrated that the KS1/4 and L-KS MoAbs recognized glycoprotein antigens of similar molecular mass and that control MoAbs had no detectable antigen-binding capability. The major band that was immunoprecipitated by the KS1/4 and L-KS MoAbs had an apparent molecular mass of 42 kDa, which is in good agreement with the results reported by Varki et al. [30]. A minor component of 35 kDa was also observed in the immunoprecipitation experiment (Fig. 1). The origin of this band is unknown but it was observed in all the KS1/4 and L-KS samples. It is possible that the 35-kDa component represents a degradation product or a biosynthetic precursor of the 42-kDa band. These data strongly suggest that the L-KS MoAbs all bind to the same epitope recognized by the KS1/4 antibody.

Large quantities of L-KS MoAbs were obtained from ascites fluid produced in pristane-primed Balb/c mice. The L1-KS MoAb was also produced in vitro in serum-free medium. The resultant immunoglobulins were purified by protein-A affinity chromatography and drug conjugates were constructed with DAVHYD. The MoAb-drug conjugates had essentially complete retention of immunological reactivity as compared to the unconjugated antibodies, and the aggregate content was less than 20%. A representative example of the antigen-binding capacity and aggregate content of an L-KS–DAVHYD conjugate is shown in Fig. 2. The top panel if Fig. 2 shows the immunological reactivity of unmodified or DAVHYD-conjugated L4-KS against UCLA-P3 lung adenocarcinoma cells. No significant difference was observed in antigen binding by free or drug-conjugated L4-KS. The bottom of Fig. 2 shows the Superose 12 profile of L4-KS–DAVHYD, which is essentially free of aggregates. The Superose 12 profile of native L4-KS is indistinguishable from the conjugate (data not shown).

The MoAb-drug conjugates thus prepared were examined for in vivo antitumor activity using several nude mouse xenograft models. The data in Fig. 3 show the tumor-suppressive activity of the L-KS–DAVHYD conjugates against 48-h-established UCLA-P3 lung tumor xenografts growing in nude mice. The data indicate that the three antibodies demonstrated good tumor-suppressive activity when conjugated to DAVHYD and that this effect was dose-dependent. It also appears from this study that antibody subisotype did not contribute significantly to the antitumor activity of the conjugates. Antibody subisotype has been implicated in the antitumor activity of some unmodified murine MoAbs [8, 9, 26]. Several studies have shown that IgG_{2a} MoAbs bind to Fc receptors of macrophages, which can result in tumor cell killing [8, 26]. No significant antitumor activity was observed for any of the unconjugated L-KS MoAbs when administered to UCLA-P3 tumor-bearing nude mice on days 2,5, and 8 after tumor cell implantation at doses as high as 150 mg/kg (data not shown). The lack of antitumor activity by the L-KS MoAbs suggests that the similar in vivo efficacy of the L-KS–DAVHYD conjugates (Fig. 3) is due to targeting of the DAVHYD to the tumor membrane and does not involve effector cell function. Since L1-, L2-, and L4-KS were as effective as MoAb-DAVHYD conjugates, subsequent experiments utilized the particular L-KS MoAb-vinca conjugate which was in greatest supply at that time. Fig. 4 shows the tumor-suppressive activity of

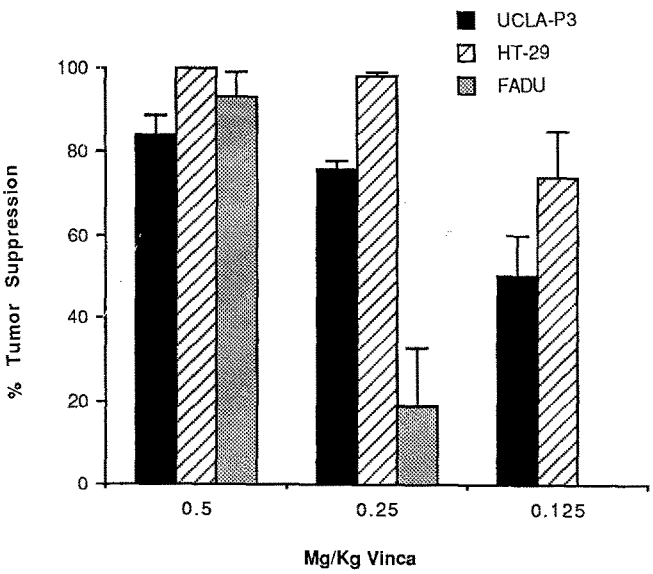

Fig. 4. In vivo activity of L1-KS–DAVHYD against three different human tumor nude mouse xenografts. Tumor cells (1×10^7) were implanted subcutaneously in nude mice and conjugate was given intravenously on days 2, 4, 7 for UCLA-P3 lung adenocarcinoma; 2, 5, 8 for HT-29 colon carcinoma; and 3, 5, 7 for FADU pharyngeal squamous carcinoma. Definitions as in legend to Fig. 3

L1-KS–DAVHYD on three human tumor nude mouse xenograft models: UCLA-P3 (lung), HT-29 (colon), and FADU (pharyngeal). These tumors were allowed to establish for 48–72 h and three conjugate treatments were administered between and 2–8 days after implantation. These data demonstrate that strong suppression was seen for all three tumors at the 0.5 mg/kg dose (vinca content) but that the FADU squamous pharyngeal carcinoma was not suppressed at the 0.25 mg/kg or 0.125 mg/kg treatments. The growth of UCLA-P3 and HT-29, on the other hand, was substantially inhibited at both the 0.25 mg/kg and 0.125 mg/kg doses (Fig. 4). These results indicated that these tumors exhibited differential in vivo sensitivity to the L1-KS–DAVHYD conjugate. One possible explantation for this observation is different KS1/4 antigen expression on the tumor cell lines. The level of KS1/4 antigen present on the UCLA-P3 and FADU human tumor

Table 3. Variable L-KS antigen expression on the UCLA-P3 and FADU human tumor cell lines[a]

	UCLA-P3		FADU	
MoAb	MFI	LEU	MFI	LEU
L1-KS	195	200	138	42
L2-KS	194	195	146	52
L4-KS	202	240	150	59
Myeloma	28	2	32	2

[a] Target cells were trypsinized and washed in tissue-culture medium. Cells were incubated in medium 1–3 h at 37°C prior to incubation with 5 μg/ml L-KS MoAb or control myeloma IgG. Mouse IgG bound to the target cells was detected by anti-(mouse IgG)-fluorescein-isothiocyanate conjugate. Mean fluorescence intensities (MFI) were determined with an Epics V flow cytometer. Linear equivalent units (LEU) were calculated by multiplying the MFI by 0.0118 and taking the antilog

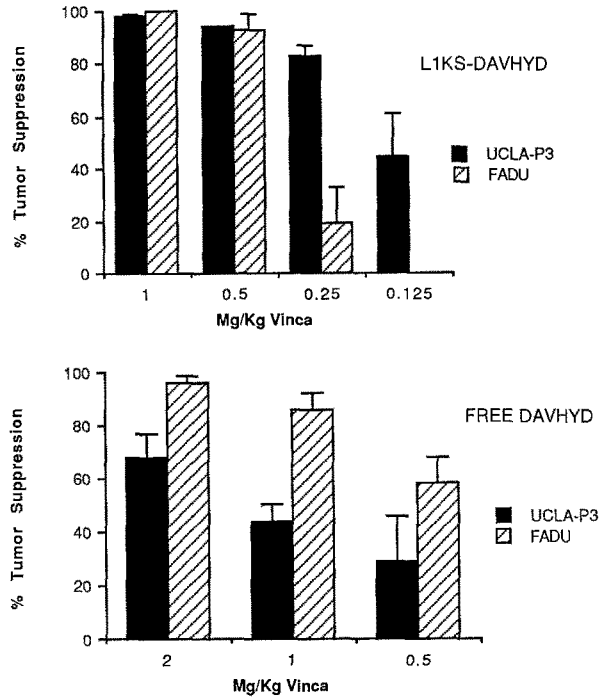

Fig. 5. In vivo activity of free or MoAb-conjugated DAVHYD against UCLA-P3 and FADU nude mouse xenografts. Tumor cells (1×10^7) were implanted subcutaneously into nude mice and conjugate or free drug was administered on days 3, 5, and 7 after implantation. Definitions as in legend to Fig. 3

cell lines was therefore quantified by flow cytometry (Table 3). L1-KS has an 80% lower surface binding to FADU (based on linear equivalent units) as compared to UCLA-P3. These cell lines were utilized as nude mouse xenografts in a direct comparison to determine the relative tumor suppressive activity of L1KS–DAVHYD and free DAVHYD. These results are shown in Fig. 5. These data show that the conjugate is efficacious against both tumors at the 1 mg/kg and 0.5 mg/kg dose (vinca content). FADU, however, is not suppressed at 0.25 mg/kg or lower doses of conjugate while UCLA-P3 is strongly inhibited at the 0.25 mg/kg dose. Although FADU is less sensitive to the conjugate, it is more strongly suppressed by the free vinca than UCLA-P3 (Fig. 3, bottom). The reduced activity by L1-KS–DAVHYD against FADU was therefore not due to an inherent resistance to the free drug by this cell line. These results strongly suggest that conjugate activity can be directly correlated with antigen expression and that the conjugate is acting in a specific manner since the tumor system that is less responsive to conjugate is more affected by the free drug.

The above conclusion that the MoAb-DAVHYD in vivo antitumor activity is antigen-mediated is also supported by the data shown in Fig. 6. In this experiment nude mice were simultaneously implanted with antigen-positive (UCLA-P3) and negative (M-14) cells in opposite flanks. As shown in Fig. 6, UCLA-P3 tumor growth was suppressed throughout the dose-response curve while M-14 was affected at only the 0.5 mg/kg dose. Utilization of free drug in the dual-flank model, on the other hand, resulted in stronger suppression against M-14 than UCLA-P3 (Fig. 6, bottom). These data again strongly suggest that the in vivo activity of L-KS–DAVHYD conjugates is an anti-

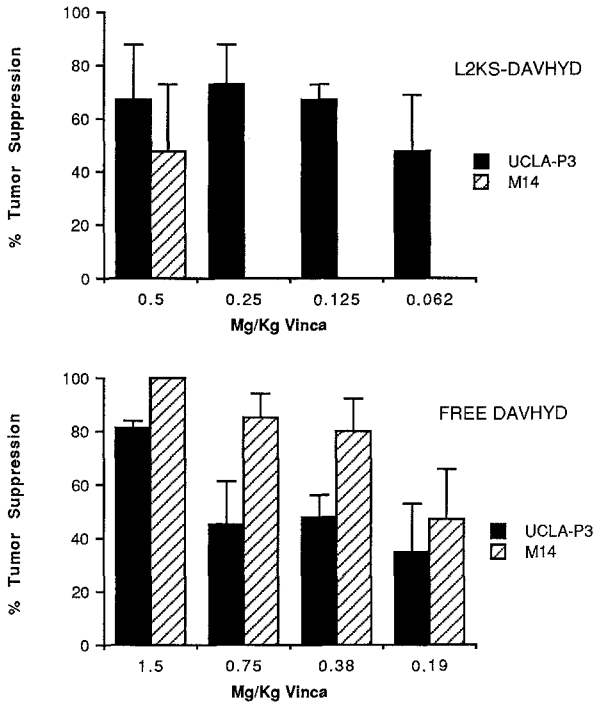

Fig. 6. Effect of free or MoAb-conjugated DAVHYD on antigen-positive or negative in a dual flank nude mouse xenograft model. Tumor cells (1×10^7) were injected subcutaneously into opposite flanks of nude mice. L2KS–DAVHYD was given intravenously on days 2, 5, 8 while free DAVHYD was administered on days 3, 5, 7 after tumor implantation. Definitions as in legend to Fig. 3

gen-dependent process and the relative lack of conjugate activity against M-14 melanoma is not due to resistance to the free drug.

The ability of L1-KS–DAVHYD and free DAVHYD to cause regression of well-established UCLA-P3 human lung tumor xenografts is shown in Fig. 7. Panels A, B, C, and D compare the effect of 2, 1, 0.5, or 0.25 mg/kg vinca content of L1-KS–DAVHYD to free DAVHYD, respectively. Significant regression of the solid tumors was accomplished with four treatments of the antibody-drug conjugate at 2, 1, and 0.5 mg/kg vinca content. The mean tumor masses at the beginning of therapy (14 days postimplantation) were 275, 353, or 395 mg for the 2, 1, or 0.5 mg/kg doses of L1-KS–DAVHYD, respectively. At day 28 following four treatments with conjugate the tumor masses had regressed to 45, 40, or 158 mg, respectively. Free DAVHYD, on the other hand, was not able to cause regression at any dose tested. Treatment of the tumor xenografts with 0.25 mg/kg vinca content of L1-KS–DAVHYD did not induce regression of the tumors but did result in suppression of tumor growth at 28-days (Fig. 7D). In contrast, free DAVHYD did not suppress tumor growth at this dose. It is also apparent from Fig. 7 that L1-KS–DAVHYD was more efficacious than free DAVHYD at every dose tested. It was also evident that the duration of tumor regression was dependent on conjugate dose. No new tumor growth was seen in the high-dose conjugate group until day 49 (Fig. 7A) whereas a rebound of tumor growth was observed at the 1 mg/kg vinca content treatment of conjugate on day 42 (Fig. 7B). Correspondingly, the tumors began to grow again on day 35 for

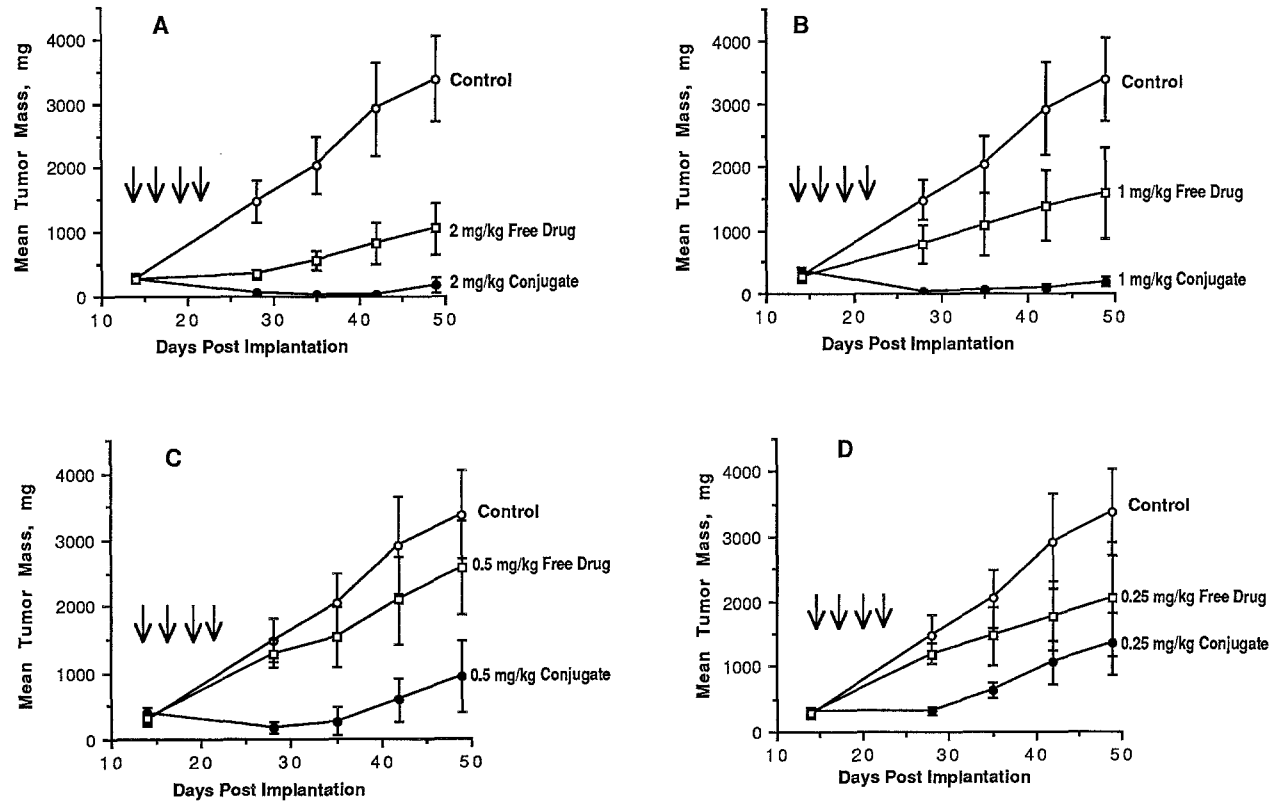

Fig. 7. Effect of free or MoAb-conjugated DAVHYD against 14-day-established UCLA-P3 nude mouse xenografts. Tumor cells 1×10^7 were implanted subcutaneously and L1-KS–DAVHYD or free DAVHYD was given intravenously on days 14, 16, 19 and 22 after tumor implantation. The ordinate is mean tumor mass in mg while the abscissa is days post implantation. Standard error bars are shown for each group. **A** 2 mg/kg vinca content dose; **B** 1 mg/kg vinca content dose; **C** 0.5 mg/kg vinca content dose; **D** 0.25 mg/kg vinca content dose

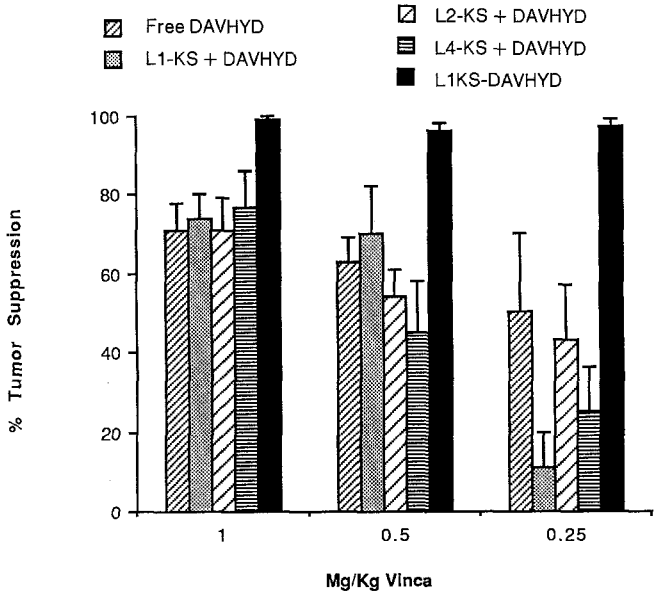

Fig. 8. Effect of mixtures of free L-KS MoAbs and free DAVHYD on UCLA-P3 nude mouse xenografts. Tumor cells (1×10^7) were implanted subcutaneously into nude mice. Mixtures of free drug and free MoAbs or 1-KS–DAVHYD were given on days 2, 5, and 8 after tumor implantation. The antibody dose used was 75 mg/kg, which corresponds to the amount of antibody given as a conjugate to achieve a 1–2 mg/kg dose of DAVHYD in the experiments described above. Definitions as in legend to Fig. 3

both the 0.5 and 0.25 mg/kg vinca content doses of L1KS–DAVHYD (Fig. 7C, D). Experiments are in progress to examine the effect of conjugate treatment on tumor cells that have reinitiated growth following conjugate-mediated regression. The above data indicate that the MoAb-DAVHYD conjugate is significantly more efficacious than the free drug as a therapeutic agent against the human lung tumor xenograft. The increase in therapeutic efficacy by the MoAb-DAVHYD conjugate was not due to sensitization of the tumor to the free DAVHYD following antibody binding to the tumor cell membrane. The data in Fig. 8 show that mixtures of free L-KSMoAbs with free DAVHYD were no more active than free drug alone and the L1-KS–DAVHYD conjugate was clearly superior to both free drug and the MoAb plus drug mixtures in this treatment regimen.

In summary, we have demonstrated that a panel of MoAbs reactive with the KS1/4 tumor-associated antigen could be conjugated with DAVHYD in such a way that the antigen-binding characteristics of the antibodies were not significantly altered. The L-KS–DAVHYD drug conjugates were efficacious in vivo against several human tumor nude mouse xenografts. The anti-tumor activity of the MoAb-DAVHYD immunoconjugates was antigen-mediated and was not significantly influenced by antibody subisotype. The MoAb-drug conjugates appeared to be more efficacious than free drug in that L1-KS–DAVHYD was able significantly to regress well-established tumors while free DAVHYD was only able to suppress tumor growth transiently.

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