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In Vivo Antitumor Activity of a Monoclonal Antibody-*Vinca* Alkaloid Immunoconjugate Directed against a Solid Tumor Membrane Antigen Characterized by Heterogeneous Expression and Noninternalization of Antibody-Antigen Complexes

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ABSTRACT

It is widely believed that antigen heterogeneity and noninternalization of antigen-antibody complexes will severely limit the antitumor activity of monoclonal antibody-drug conjugates. The B72.3 monoclonal antibody binds to a tumor-associated antigen which is heterogeneously expressed in human carcinomas (J. Schlom, Cancer Res., *46:* 3225–3238, 1986). We therefore performed studies to assess the degree of internalization of B72.3 antibody-antigen complexes and the level of *in vivo* antitumor activity that could be achieved with B72.3 conjugated to 4-desacetyl vinblastine-3-carboxhydrazide. Internalization studies were performed on LS174T colorectal carcinoma and OVCAR-3 ovarian carcinoma cells using iodinated B72.3 as well as an iodinated antibody that binds to the human transferrin receptor, HB-21. These data indicated that, in contrast to HB-21, the B72.3 antigen-antibody complex was not internalized. The B72.3-*Vinca* alkaloid immunoconjugate demonstrated significant antitumor activity against LS174T xenografts, although complete regressions of established tumors were not achieved. Immunohistochemical analyses indicated that the B72.3 antigen was heterogeneously expressed in the LS174T xenografts and that tumor cells which were not killed by high doses of B72.3-*Vinca* also expressed the B72.3 antigen. These studies indicated that significant antitumor activity may be achieved by monoclonal antibody-drug conjugates even when antigen heterogeneity and noninternalization of antigen-antibody complexes are encountered. The data also suggested that the formulation of antibody-drug conjugate cocktails to counteract antigen heterogeneity may not be sufficient to eradicate all malignant cells within a solid tumor mass.

INTRODUCTION

One of the most commonly cited theoretical limitations to the potential usefulness of MoAb[2]-drug conjugates as effective oncolytic agents is the necessity of conjugate internalization for cytotoxic activity (1–4). The observation that many solid tumor membrane antigens are stable cell surface components suggests that MoAb-drug conjugates may be ineffective against these target antigens (3). The phenomenon of antigen heterogeneity is also an important consideration which has led to the prediction that antigen-negative tumor cells will not bind the immunoconjugate and thereby escape conjugate-mediated cytotoxicity. In spite of these theoretical limitations, a number of laboratories have reported success in the construction of MoAb-drug conjugates effective against solid human tumor xenografts (5–12). Several of these reports (10–12) have focused on MoAb-drug conjugates directed against the KS1/4 adenocarcinoma-associated antigen (13, 14). The KS1/4 antigen is homogeneously expressed on a number of human tumors and normal tissues (14), and the structure deduced from its amino acid sequence is that of a transmembrane glycoprotein (15). The biosynthesis and glycosylation pattern of this molecule have been recently described (16). Previous data from our laboratory (17) indicated that the KS1/4 antigen does not rapidly internalize following immunoconjugate localization. This observation led to the postulate that the labile nature of the chemical bond used to construct the MoAb-*Vinca* conjugate in the above study (18) may have contributed to the cytotoxicity of the immunoconjugate by releasing the drug at the periphery of the tumor cell (thereby allowing transport into the cell) following *in vivo* localization (17). If this hypothesis is correct, it may be possible to prepare active MoAb-drug conjugates reactive with noninternalizing and heterogeneously expressed solid tumor membrane antigens. The B72.3 MoAb binds to a high-molecular-weight glycoprotein, TAG-72, which is expressed in tumors of the human colon, lung, ovary, breast, pancreas, and prostate (3, 19–23). A hallmark of the TAG-72 antigen is its heterogeneous expression in the above tumors (3, 19–23). Thus, while the antigen is expressed in a high percentage of human adenocarcinomas, the percentage of reactive cells can range from 5 to >95% (3, 19–23). Preclinical and clinical imaging studies have demonstrated that the B72.3 MoAb remains at the tumor site for a number of days following localization (24, 25), which indicates that B72.3 forms a stable complex with the TAG-72 antigen *in vivo*. We therefore felt that the TAG-72 antigen represented a stringent test as a target for MoAb-drug conjugate immunotherapy and would help to elucidate the relative importance of antigen heterogeneity on the escape of antigen-negative tumor cells from site-directed therapy.

MATERIALS AND METHODS

Chemicals and Reagents. Rabbit anti-mouse IgG-fluorescein isothiocyanate was purchased from Jackson ImmunoResearch Laboratories, Avondale, PA. DMEM, γ-globulin-free horse serum, trypsin-EDTA, L-glutamine, and gentamicin were obtained from GIBCO Laboratories, Gaithersburg, MD. Fetal calf serum was supplied by Hyclone Laboratories, Logan, UT. $Na^{125}I$ was from DuPont NEN Research Products, Boston, MA, and Iodo-Beads were purchased from Pierce Chemical Co., Rockford, IL. Biotinyl *N*-hydroxysuccinimide ester was from E-Y Labs., Inc., San Mateo, CA.

Monoclonal Antibodies and Immunoconjugates. The hybridoma cell line secreting the B72.3 IgG1 MoAb was obtained from Dr. Richard Bartholomew, Hybritech, Inc., San Diego, CA. The cells were grown as ascites, and the B72.3 MoAb was purified from ascites fluid using Protein A affinity chromatography (26). Non-antigen-binding MoAbs of the IgG1 and IgG2b subisotypes were also purified from ascites fluid using Protein A chromatography. The HB21 hybridoma was obtained from the American Type Culture Collection, Rockville, MD. The IgG1

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[2] The abbreviations used are: MoAb, monoclonal antibody; DAVLBHYD, 4-desacetyl vinblastine-3-carboxhydrazide; DMEM, Dulbecco's modified Eagle's medium; IgG, immunoglobulin G; IgM, immunoglobulin M.

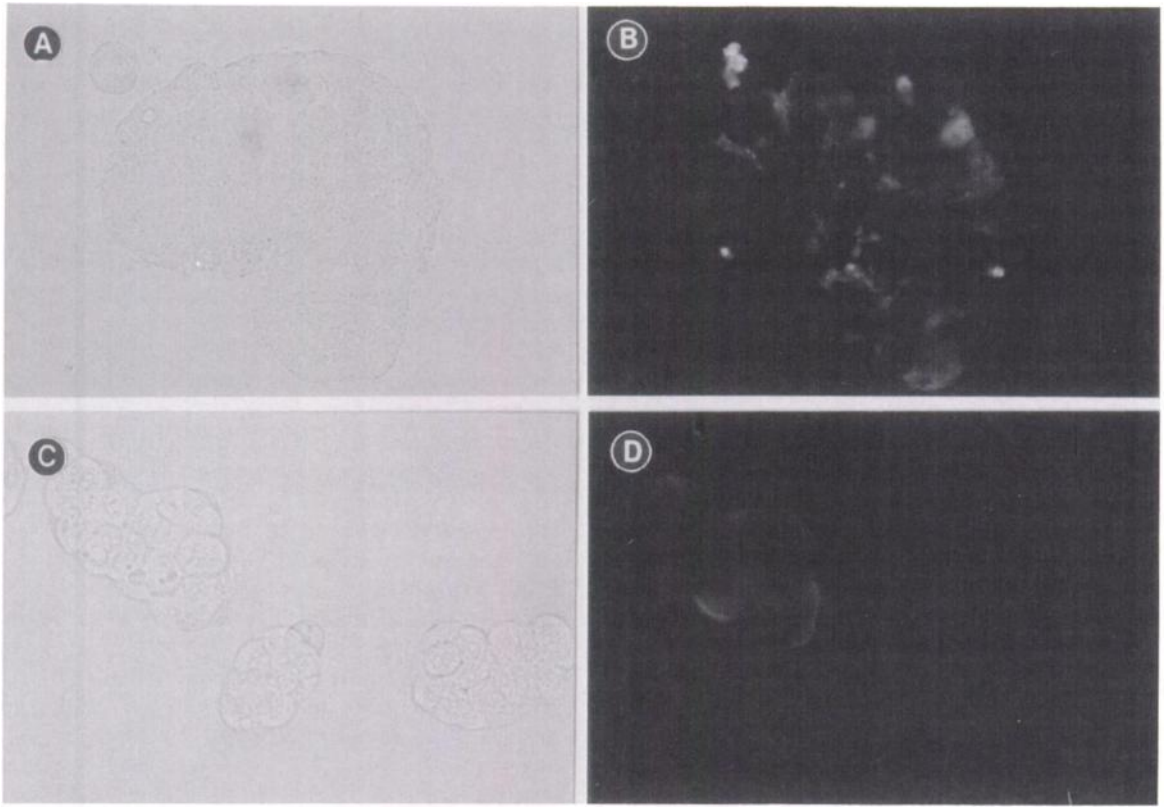


Fig. 1. Heterogeneous expression of the TAG-72 antigen on LS174T and OVCAR-3 carcinoma cells. *A* and *C* are bright-field photographs of LS174T and OVCAR-3 carcinoma cells, respectively. *B* and *D* are immunofluorescent micrographs of B72.3 MoAb membrane reactivity with LS174T and OVCAR-3 tumor cells, respectively. Immunofluorescence using a subisotype-matched irrelevant IgG1 control was negative. × 300.

MoAb secreted by this hybridoma reacts with the human transferrin receptor (27). Purified HB21 MoAb was a gift from Dr. Leroy Baker, Lilly Research Laboratories, Indianapolis, IN. Purified B72.3 and irrelevant MoAbs were conjugated to DAVLBHYD as previously described (18). Briefly, MoAbs were concentrated to about 10 mg/ml by vacuum dialysis in phosphate-buffered saline and subsequently dialyzed against 0.1 M sodium acetate, pH 5.6. The antibodies were oxidized by treatment with 160 mM sodium metaperiodate and purified by Sephadex G-25 chromatography in the acetate buffer. Conjugation to DAVLBHYD 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 gel chromatography. This procedure routinely resulted in the conjugation of 4 to 6 mol of drug per mol of MoAb. Immunoreactivity of MoAbs and MoAb-drug conjugates was determined by a solid-phase radioimmunoassay (12) against LS174T human colon adenocarcinoma cells. Purified 9.2.27 IgG2a anti-melanoma MoAb (28) was kindly provided by Dr. Thomas F. Bumol, Lilly Research Laboratories.

Human Tumor Cell Lines. LS174T colorectal carcinoma cells were purchased from the American Type Culture Collection and propagated *in vitro* in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, L-glutamine, and 50 μg/ml of gentamicin. OVCAR-3 ovarian carcinoma cells (29, 30) were provided by Dr. Thomas F. Bumol, Lilly Research Laboratories, and maintained as an ascites in nude mice.

Immunofluorescence. OVCAR-3 cells were removed from the peritoneal cavity of a nude mouse and resuspended at a 1:5 dilution in DMEM containing 10% fetal calf serum. The tumor cells were allowed to settle at 1 × *g*, the supernatant was discarded, and the pellet was washed twice in cold DMEM plus 10% fetal calf serum. LS174T colon carcinoma cells were removed from the tissue culture substratum using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing EDTA and glucose (12). The LS174T cells were washed twice in medium, and both cell lines were resuspended in cold DMEM plus 10% fetal calf serum to a final concentration of 5×10^5 to 1×10^6 cells/ml. One-ml aliquots were placed in centrifuge tubes, and the suspensions were centrifuged at 200 × *g* for 5 min. The supernatants were discarded, and the pellets were resuspended in 0.1 ml of cold medium containing 10 μg/ml of B72.3 or irrelevant IgG1 MoAb. The cells were incubated for 1 h at 4°C and washed twice with cold medium. The pellets were resuspended in 0.1 ml of a 1:100 dilution of rabbit anti-mouse IgG-fluorescein isothiocyanate in cold medium and incubated another hour at 4°C. The cells were washed twice in cold medium, and wet mounts were prepared for examination by fluorescent microscopy.

Immunohistochemistry. Tumors were excised from the flanks of nude mice, cut into 2-mm cubes, covered with embedding medium, and snap frozen in liquid nitrogen. Frozen sections were fixed in acetone for 15 min and allowed to air dry. Endogenous peroxidase was blocked by adding 0.3% H_2O_2 to rehydrated sections. The slides were washed, and 10 μg/ml of biotinylated MoAb (biotinylated using biotinyl *N*-hydroxysuccinimide ester according to the procedure outlined in Ref. 31) were added to the sections for 1 h at room temperature. The slides were washed, and antibody binding was detected using streptavidin-horseradish peroxidase followed by diaminobenzidine treatment. The sections were counterstained with hematoxylin, dehydrated, and mounted with Permount for microscopical examination.

Internalization Assays. Internalization studies were performed using acid dissociation of iodinated MoAbs as described previously (17). Briefly, LS174T human colorectal carcinoma cells or OVCAR-3 human ovarian carcinoma cells were harvested as described above for immunofluorescence and resuspended to 5×10^5 to 1×10^6 cells/ml in complete DMEM. The cells were incubated on ice for 30 min in the presence of 1×10^6 cpm of ^{125}I-B72.3 or ^{125}I-HB21 [MoAbs were labeled by the Iodo-Bead method (32) to a specific activity of 1 to 3×10^6 cpm/μg]. Excess antibody was washed away, and the cells were incubated at 37°C for various times in tissue culture medium. After centrifugation, the pellets were counted in a gamma counter. ^{125}I-B72.3 spontaneously released from LS174T tumor cells as a function of incubation at 37°C was evaluated for the presence of high-molecular-weight antigen-antibody complexes by Sephadex G200 gel chromatography. Acid dissociation experiments were performed by treating the

labeled cells for 15 min with 1 ml of glycine buffer [0.05 M glycine-HCl (pH 2.8):0.1 M NaCl; Ref. 1]. Acid-resistant cpm remaining in the cell pellets were determined in a gamma counter.

Affinity Constant Determinations. LS174T tumor cells were harvested as described for immunofluorescence. Cells (1.25×10^7) were placed in test tubes and equilibrated to 4°C or 37°C. ^{125}I-B72.3 was added (30,000 cpm; specific activity, 1.5×10^6 cpm/μg; 50% immunoreactive antibody) in the presence of varying amounts of competing nonradioactive B72.3 MoAb. Maximal binding occurred in 1 h or 15 min at 4°C or 37°C, respectively. Affinity constants of ^{125}I-B72.3 binding to LS174T tumor cells at 4°C and 37°C were determined by the method of Scatchard (33).

Nude Mouse Xenograft. LS174T tumor cells were grown *in vitro*, removed from the substratum, and washed 3 times in phosphate-buffered saline, and 1×10^7 cells were injected s.c. in the hindquarters of 25- to 28-g female outbred nude mice (Charles River Breeding Laboratories, Boston, MA). The tumors were allowed to establish for various times, and treatments of phosphate-buffered saline, MoAb-DAVLBHYD conjugates, and free DAVLBHYD were given i.v. at defined intervals. Tumor size was determined by caliper measurements, and tumor mass in mg was estimated from the formula $l \times w^2/2$, where l is the larger and w is the smaller of perpendicular diameters. All control groups contained 10 animals, whereas treatment groups comprised 5 mice each.

RESULTS

Cell surface expression of the TAG72 antigen on LS174T (Fig. 1, *A* and *B*) and OVCAR-3 (Fig. 1, *C* and *D*) is shown.

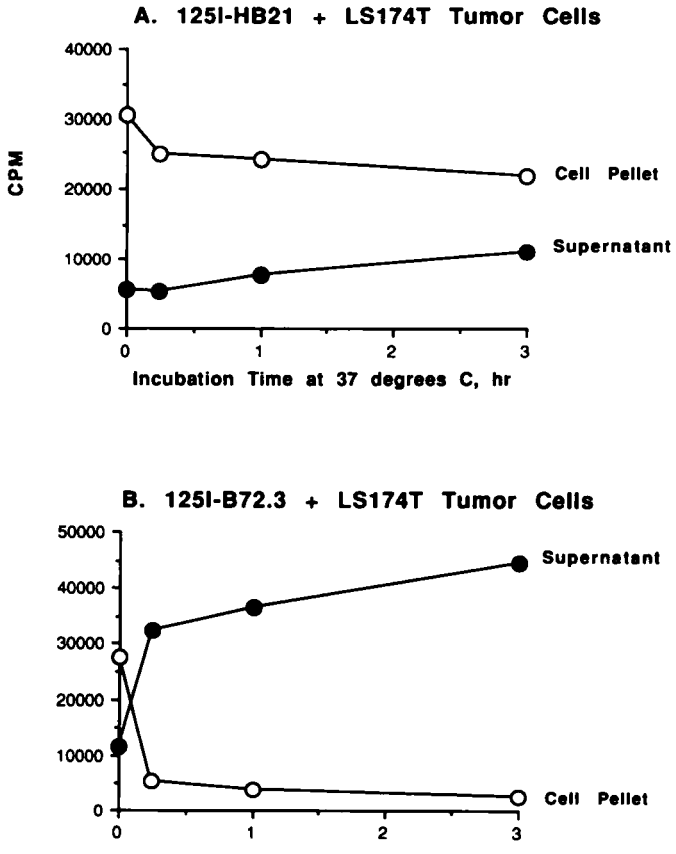


Fig. 2. Stability of iodinated MoAbs on LS174T tumor cells as a function of incubation at 37°C. ^{125}I-HB21 (*A*) or ^{125}I-B72.3 (*B*) was bound to LS174T tumor cells at 0°C. Excess MoAb was removed, and cells were incubated at 37°C for various times. Cells were centrifuged, and cpm remaining in the pellets as well as cpm released into the supernatant were determined in a gamma counter. Negligible cpm remained soluble after trichloroacetic acid precipitation (10%, v/v) with either antibody.

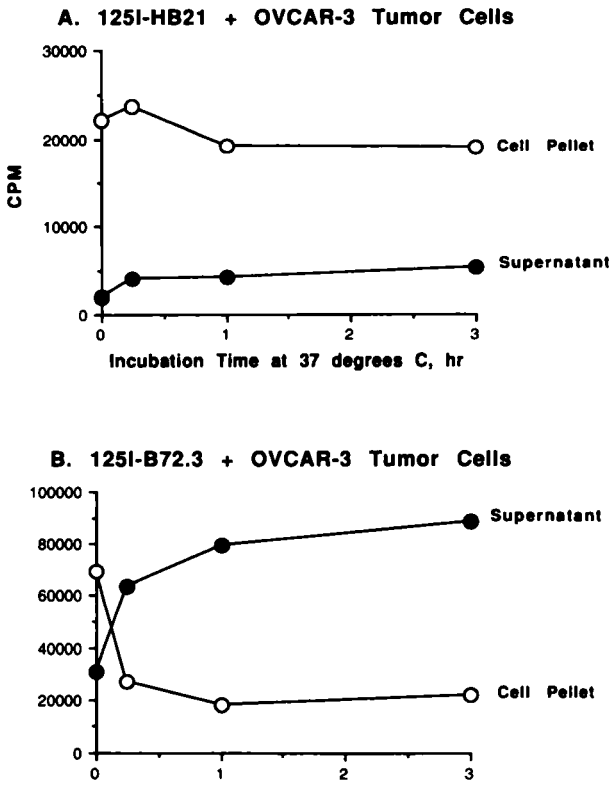
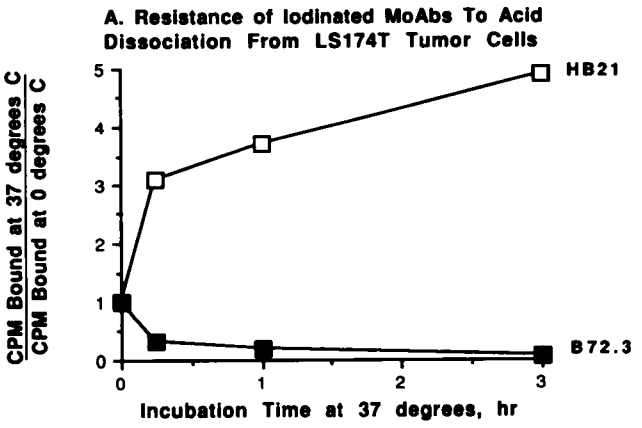


Fig. 3. Stability of iodinated MoAbs on OVCAR-3 tumor cells as a function of incubation at 37°C. ^{125}I-HB21 (*A*) or ^{125}I-B72.3 (*B*) was bound to OVCAR-3 tumor cells at 0°C. Excess MoAb was removed, and cells were incubated at 37°C for various times. Cells were centrifuged, and cpm remaining in the pellets as well as cpm released into the supernatant were determined in a gamma counter. Negligible cpm remained soluble after trichloroacetic acid precipitation (10%, v/v) with either antibody.

The B72.3 MoAb exhibits heterogeneous reactivity with both tumor lines as determined by immunofluorescence. These data are consistent with the published results of Thor *et al.* (19) and Schlom *et al.* (34).

Internalization of B72.3 after binding to LS174T or OVCAR-3 tumor cells was examined utilizing iodinated MoAb in an acid dissociation assay (17). The assumption inherent in this experimental approach (1) is that ^{125}I-B72.3 bound to cells at 0°C will become resistant to acid dissociation if the antibody-antigen complex is internalized after warming the cells to 37°C. ^{125}I-HB21, an antibody that recognizes the transferrin receptor, is included in the assay as a positive control for internalization. As a prelude to the acid dissociation assay, the stability of the ^{125}I-MoAb bound to the target cell at 0°C is determined after warming the cells to 37°C to ascertain if antibody is being spontaneously released from the cells as a function of the higher temperature. The data shown in Figs. 2*A* and 3*A* demonstrate that ^{125}I-HB21 is relatively stable on both LS174T and OVCAR-3 tumor cells. The slow release of cpm from the cell pellets and corresponding increase in supernatant cpm may be due to externalization of the transferrin receptor-HB21 complex into the medium (35). ^{125}I-B72.3, on the other hand, is rapidly released into the supernatant by both tumor cell lines after warming the cells to 37°C (Figs. 2*B* and 3*B*). The data in Fig. 4, *A* and *B*, demonstrate that ^{125}I-HB21 becomes resistant to acid dissociation from both LS174T and OVCAR-3 tumor cells after only a 15-min incubation at 37°C, which is indicative of a rapidly internalizing antibody-antigen complex. ^{125}I-B72.3 does not become resistant to acid dissociation, however, and in fact the ratio of cpm bound at 37°C to cpm bound at 0°C



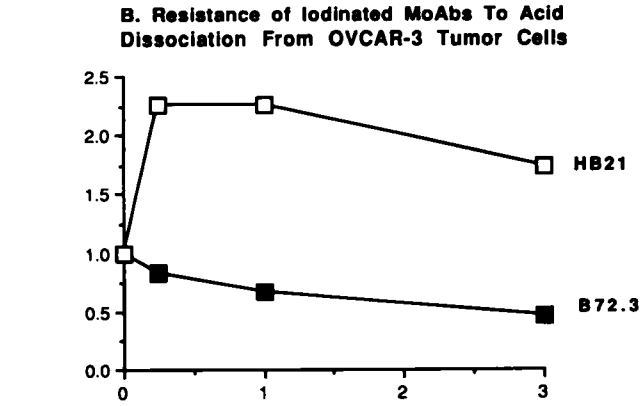


Fig. 4. Resistance of iodinated MoAbs to acid dissociation. LS174T (*A*) or OVCAR-3 (*B*) tumor cells were labeled with iodinated MoAbs at 4°C. After incubation at 37°C for the indicated times, the cells were treated with a glycine-HCl buffer, pH 2.8. Cell-bound cpm were determined by a gamma counter.

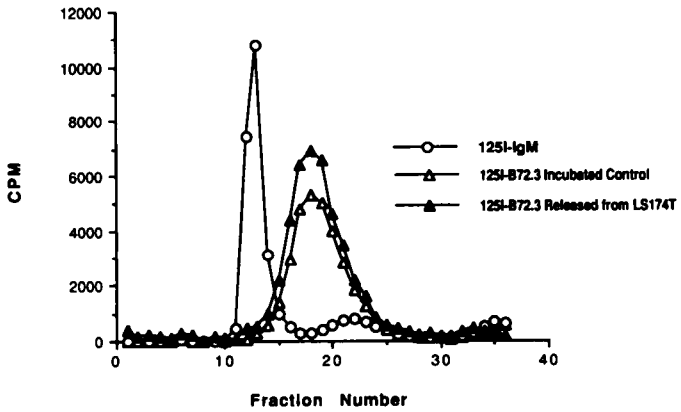


Fig. 5. Absence of ^{125}I-B72.3 MoAb-TAG72 antigen complex formation as determined by Sephadex G-200 chromatography. LS174T tumor cells were labeled with 1×10^6 cpm of ^{125}I-B72.3 at 0°C. Excess MoAb was washed away, and the cells were incubated at 37°C for 1 h. Iodinated B72.3 released from the cells was collected and applied to a 1- x 30-cm Sephadex G-200 column equilibrated in phosphate-buffered saline (10 mM PO_4:150 mM NaCl, pH 7.4) containing 0.5% bovine serum albumin and 0.05% sodium azide. ^{125}I-B72.3 incubated at 37°C in the absence of tumor cells was also chromatographed as a control. ^{125}I-IgM (purified human myeloma IgM, a gift from Dr. Victor Chen, Lilly Research Laboratories, labeled by the Iodo-Bead method, Ref. 32) was utilized to define the exclusion volume of the column. Fraction size is 0.6 ml, and actual cpm values were multiplied by appropriate factors (cpm values obtained for IgM, B72.3 incubated control, and B72.3 released from the cells were multiplied by 3, 2, and 10, respectively) to normalize samples to approximate equivalency in cpm loaded onto the column.

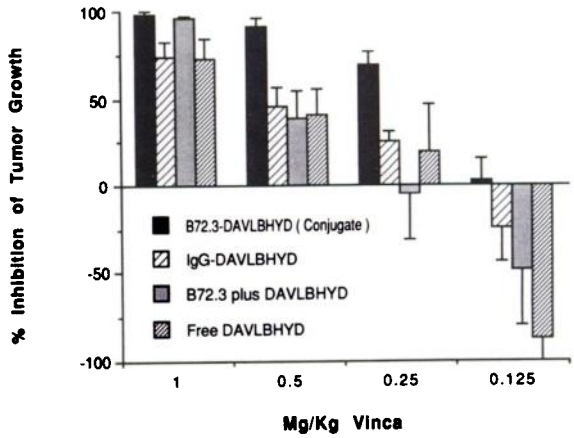


Fig. 6. *In vivo* antitumor activity of B72.3-DAVLBHYD against 2-day established LS174T tumor xenografts. Nude mice were given injections s.c. of 1×10^7 LS174T tumor cells. B72.3-DAVLBHYD, irrelevant IgG1-DAVLBHYD, B72.3 plus DAVLBHYD, or free DAVLBHYD was given i.v. at Days 2, 5, and 8 after implantation. The *abscissa* is the treatment dose given in mg/kg of *Vinca* equivalent, while the *ordinate* is tumor suppression (given as a percentage). Tumor suppression is defined as $[1 - (a/b)] \times 100$, where a is the mean tumor mass of the treatment animals, and b is the mean tumor mass of the saline-injected control group. The data shown were obtained 21 days after tumor implantation. *Columns*, mean; *bars*, SE.

actually decreases throughout the time course of the experiment (Fig. 4, *A* and *B*). The decreased ratio is probably due to the large number of cpm of ^{125}I-B72.3 spontaneously released from the tumor cell pellets at 37°C (Figs. 2*B* and 3*B*). Since the TAG-72 antigen recognized by B72.3 is a large (>1×10^6 daltons) secreted mucin (36), it was possible that the loss of iodinated B72.3 from the tumor cells at 37°C was due to the secretion of ^{125}I-B72.3-TAG-72 complexes into the tissue culture medium. The presence of such complexes was analyzed by Sephadex G-200 chromatography (Fig. 5). The data in Fig. 5 indicated that ^{125}I-B72.3 was not secreted from LS174T tumor cells as a complex with the TAG-72 antigen, since the gel chromatography profile of iodinated B72.3 released from the cells at 37°C was indistinguishable from the profile obtained with incubated control ^{125}I-B72.3. Iodinated human IgM (M_r 900,000) was also chromatographed on the Sephadex G-200 column to demonstrate that this gel system was capable of resolving any putative ^{125}I-B72.3-TAG72 complexes from uncomplexed antibody. The lack of detectable large-molecular-weight complexes was not due to inadequate sensitivity of this technique, since similar studies using second generation anti-TAG-72 MoAbs (37) could detect significant immune complex formation (38). Another possible explanation for the spontaneous release of ^{125}I-B72.3 from the tumor cell surface was a decrease in the affinity constant for this antibody at 37°C relative to 0°C. Johnstone *et al.* (39) have recently demonstrated that equilibrium constants for a panel of MoAbs can be greatly influenced by temperature and that the dissociation rate constant can be substantially increased relative to the association rate constant at higher temperatures for some MoAbs. Consistent with this observation, it was determined that the affinity constant of ^{125}I-B72.3 binding to LS174T tumor cells decreased from 6.7×10^8 M^{-1} at 4°C to 1.7×10^8 M^{-1} at 37°C (data not shown).

The *in vivo* antitumor activity of B72.3-DAVLBHYD was determined against LS174T human tumor xenografts using various dosing schedules. The B72.3-DAVLBHYD conjugates used in these studies contained 4 to 6 mol of drug bound per mol of IgG and exhibited good immunoreactivity characteris-

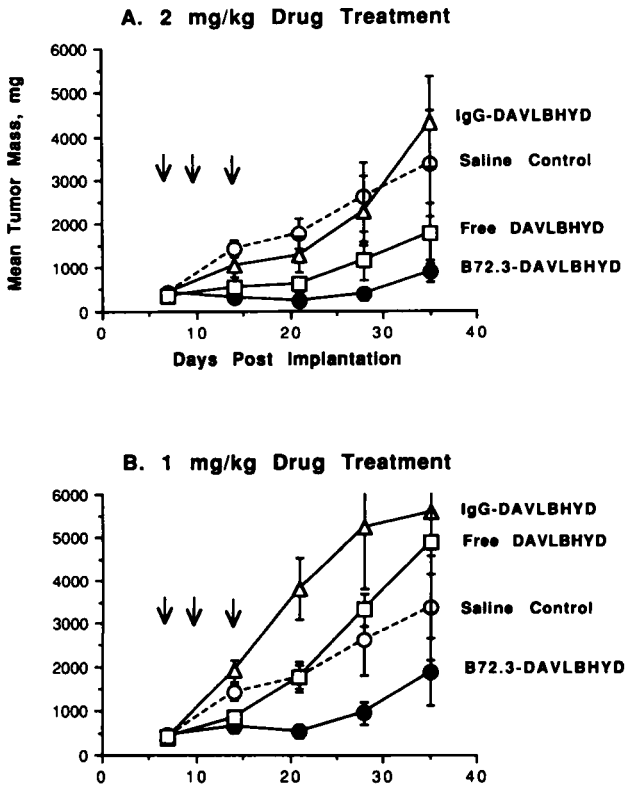


Fig. 7. *In vivo* antitumor activity of B72.3-DAVLBHYD against 7-day established LS174T tumor xenografts receiving 3 doses of conjugate. Carcinoma cells (1×10^7) were injected s.c. into nude mice. Intravenous therapy using saline, B72.3-DAVLBHYD, irrelevant IgG2b-DAVLBHYD, or unconjugated DAVLBHYD was performed on Days 7, 10, and 14. The *ordinate* is mean tumor mass in mg, while the *abscissa* is days after implantation. *Points*, mean; *bars*, SE. *A*, 2-mg/kg *Vinca* dose; *B*, 1-mg/kg *Vinca* dose.

tics as described previously (Refs. 12 and 18; data not shown). The study depicted in Fig. 6 examined the dose-response effect of B72.3-DAVLBHYD, irrelevant (nonantigen binding) IgG1-DAVLBHYD, B72.3 plus DAVLBHYD, and free DAVLBHYD on growth inhibition of 2-day established LS174T tumors. The results of this experiment indicated that all treatments strongly suppressed tumor growth at the 1-mg/kg (*Vinca* content) dose, whereas B72.3-DAVLBHYD was clearly the most efficacious therapy at 0.5 mg/kg. B72.3-DAVLBHYD also appeared to have the strongest tumor-suppressive activity at 0.25 mg/kg, while none of the treatments was effective at the 0.125-mg/kg dose (Fig. 6). It is interesting to note that irrelevant IgG1-DAVLBHYD, B72.3 plus DAVLBHYD, and free DAVLBHYD appear to enhance tumor growth at 0.125 mg/kg. The growth enhancement effect of 0.125 mg/kg of free DAVLBHYD was observed in 2 of 3 other unrelated experiments using LS174T xenografts and the same dosing schedule. The reason(s) for the increased tumor growth at this dose level for these agents is unknown. The antitumor activity of B72.3-DAVLBHYD, IgG-DAVLBHYD, and free DAVLBHYD against 7-day established LS174T tumor xenografts is shown in Figs. 7 to 9. B72.3-DAVLBHYD was superior to saline control, irrelevant IgG-DAVLBHYD, and free DAVLBHYD when given 3 times i.v. at 2 and 1 mg/kg (*Vinca* content; Fig. 7). The data in Fig. 7 also indicated that the 2-mg/kg dose of B72.3-DAVLBHYD was able to achieve a transient regression in the size of the LS174T tumors; *i.e.*, 419 ± 75 (SE) mg at the start of therapy regressed to 215 ± 53 (SE) mg at Day 21. Four i.v. administrations of 2 mg/kg (*Vinca*) of B72.3-DAVLBHYD were also more efficacious than irrelevant conjugate or free drug against 7-day established LS174T tumor xenografts (Fig. 8). The results presented in Fig. 8 also demonstrated that a 2-mg/kg dose of B72.3-DAVLBHYD could induce a transient regression of tumor size [484 ± 71 (SE) mg at initiation of treatment reduced to 299 ± 76 (SE) mg at Day 14]. Finally, an expanded dose-response utilizing 6 i.v. treatments of B72.3-DAVLBHYD, IgG1-DAVLBHYD, and free DAVLBHYD is presented in Fig. 9. The 2-mg/kg (*Vinca*) dose of B72.3-DAVLBHYD also caused a transient regression of the LS174T tumor [709 ± 113 (SE) mg at Day 7 regressed to 472 ± 119 (SE) mg at Day 14] on this dosage schedule (Fig. 9*A*). Free DAVLBHYD and irrelevant conjugate, however, exhibited comparable activity to B72.3-DAVLBHYD at the 2-mg/kg dose (Fig. 9*A*), although neither of these agents regressed the tumor. B72.3-DAVLBHYD appeared to be slightly more active than free DAVLBHYD at the 1-mg/kg dose (Fig. 9*B*), while IgG1-DAVLBHYD loses much of its antitumor activity at this dose. B72.3-DAVLBHYD is clearly the superior reagent at the 0.5-mg/kg treatment level (Fig. 9*C*). The lowest dose utilized in this experiment (0.25 mg/kg; Fig. 9*D*) did not display significant antitumor activity for any of the DAVLBHYD-containing reagents compared with the saline control-treated mice.

Tumors from the various treatment groups shown in Fig. 9 were removed from the animals 4 days after the last tumor measurement (15 days after the last i.v. treatment) and used to elucidate TAG-72 antigen expression using immunoperoxidase analysis of frozen sections. Fig. 10, *A* to *C*, shows the TAG-72 antigen expression of LS174T tumors remaining after 6 i.v. treatments with 2, 1, and 0.5 mg/kg (*Vinca*) of B72.3-DAVLBHYD, respectively. TAG72 antigen was heterogeneously expressed on sections from each dose group which indicated that treatment of LS174T tumors with B72.3-DAVLBHYD *in vivo* did not result in the eradication of antigen-positive tumor cells. Fig. 10*D* shows the lack of immunoperoxidase staining when the irrelevant biotinylated MoAb is used in the immunostaining procedure, while *E* and *F* indicate the level of TAG72 antigen expression in the 2-mg/kg free DAVLBHYD and 2-mg/kg irrelevant IgG1-DAVLBHYD treatment groups (Fig. 9*A*), respectively.

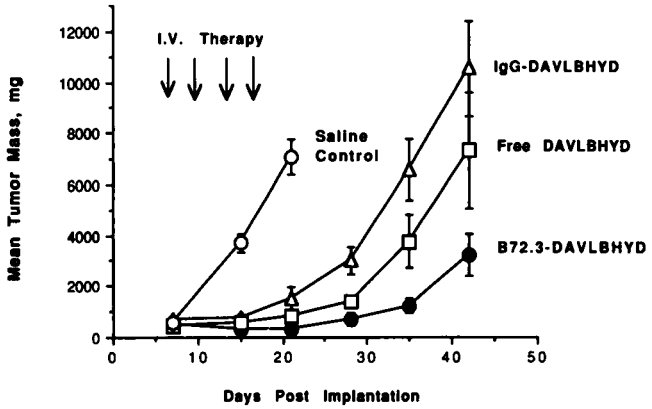


Fig. 8. Antitumor effect of B72.3-DAVLBHYD against 7-day established LS174T xenografts receiving 4 doses of conjugate. Animals were treated i.v. on Days 7, 10, 14, and 17 with 2 mg/kg of *Vinca* content of B72.3-DAVLBHYD, irrelevant IgG1-DAVLBHYD, free DAVLBHYD, or saline as indicated. The *ordinate* is the mean tumor mass in mg, while the *abscissa* is days after implantation. *Points*, mean; *bars*, SE.

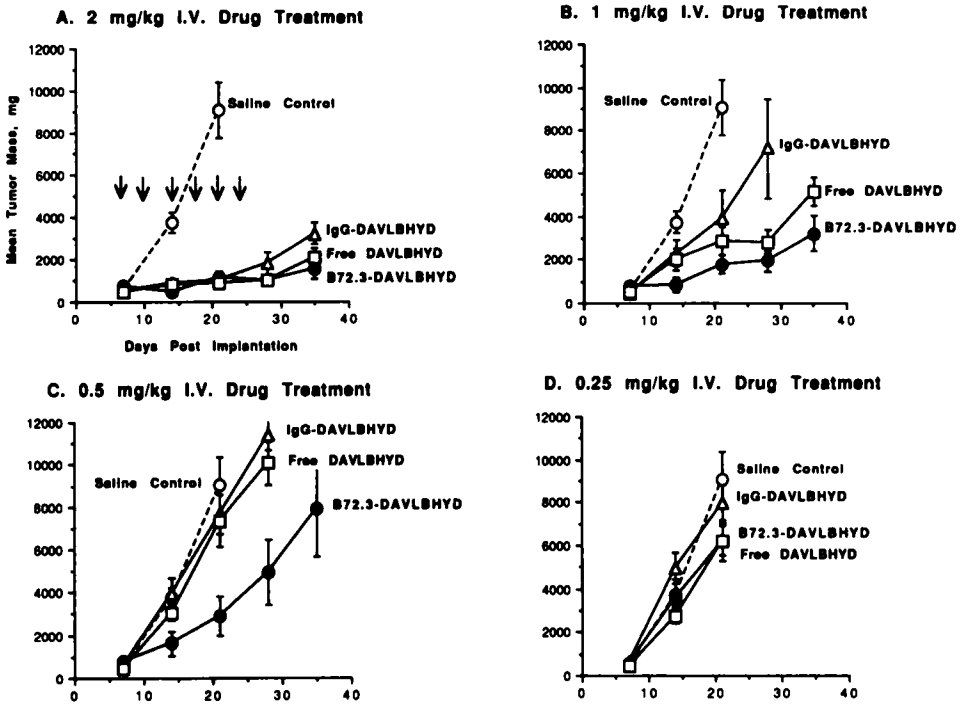


Fig. 9. *In vivo* efficacy of B72.3-DAVLBHYD against 7-day established LS174T xenografts receiving 6 doses of conjugate. Animals were treated i.v. on Days 7, 10, 14, 17, 21, and 24 with B72.3-DAVLBHYD, irrelevant IgG1-DAVLBHYD, free DAVLBHYD, or saline. The *ordinate* is the mean tumor mass in mg, while the *abscissa* is days after implantation. *Points*, mean; *bars*, SE. *A*, 2-mg/kg *Vinca* dose; *B*, 1-mg/kg *Vinca* dose; *C*, 0.5-mg/kg *Vinca* dose; *D*, 0.25-mg/kg *Vinca* dose.

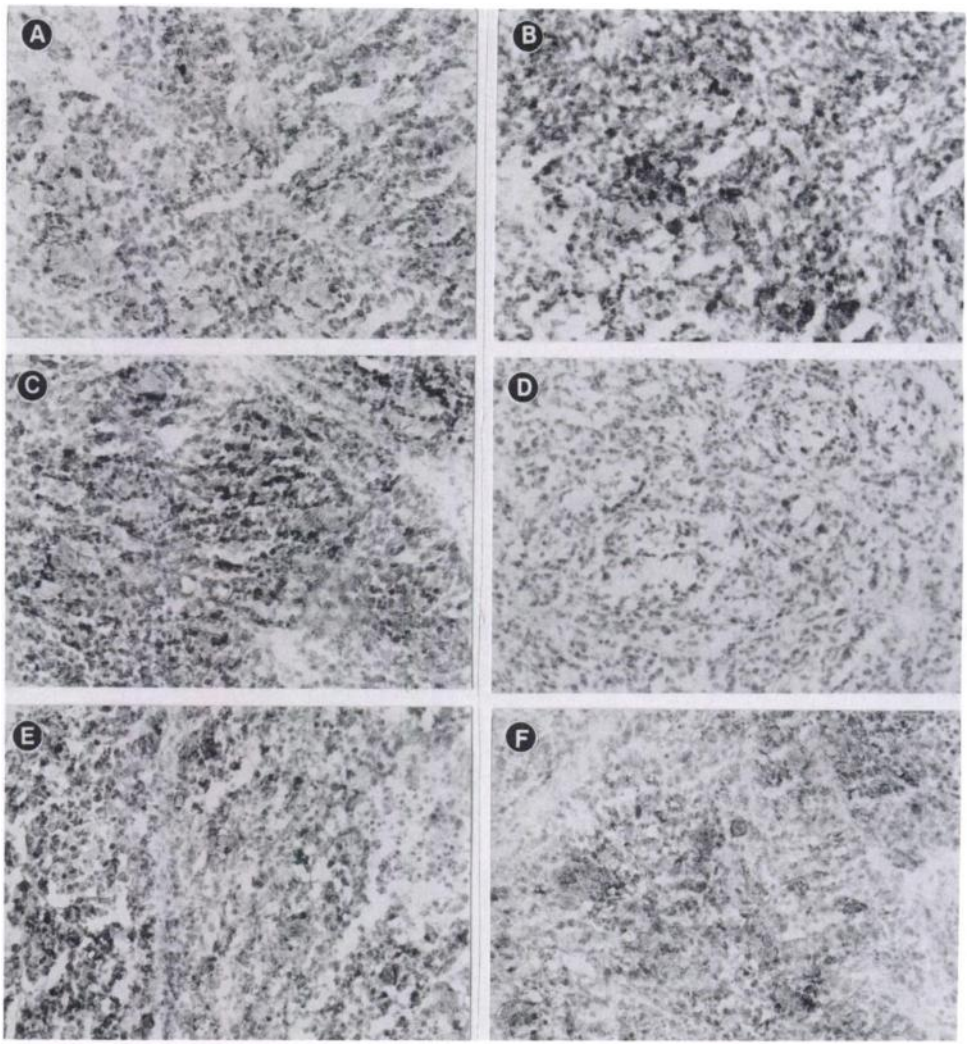


Fig. 10. TAG-72 antigen expression on LS174T tumor xenografts following *in vivo* conjugate or free drug therapy. Tumors from animals treated in Fig. 9 were removed 4 days following the last indicated measurement and processed for immunohistochemistry. TAG-72 antigen expression was determined using biotinylated B72.3 and streptavidin-horseradish peroxidase (*A* to *C*, *E*, and *F*). An irrelevant biotinylated IgG2a MoAb was used (*D*) to show specificity of the reaction. *A* to *C* represent TAG-72 antigen expression in xenografts treated with 2, 1, and 0.5 mg/kg (*Vinca*) of B72.3-DAVLBHYD. *D* is the irrelevant MoAb reacted with a tumor treated with 0.5 mg/kg of B72.3-DAVLBHYD. *E* shows TAG-72 expression in an LS174T xenograft treated with 2 mg/kg of free DAVLBHYD, while *F* demonstrates the antigen present in tissue from an animal treated with 2 mg/kg of irrelevant conjugate. × 450.

DISCUSSION

The TAG-72 antigen recognized by the B72.3 MoAb represents a difficult challenge for the construction of active chemoimmunoconjugates targeted against this antigen system. B72.3 has heterogeneous reactivity with human adenocarcinomas (Figs. 1 and 10; Refs. 3, 19–23) and does not appear to be internalized after binding to tumor cells (Fig. 4). As shown above, however, the internalization assay used in our studies which measured ^{125}I-B72.3 that is resistant to acid dissociation after incubating target cells at 37°C (Fig. 4) is somewhat complicated by the propensity of the radiolabeled antibody to spontaneously dissociate from the cells (Figs. 2 and 3). This can be explained by the decreased equilibrium constant of ^{125}I-B72.3 binding to tumor cells at 37°C compared with binding at cold temperatures (keq = 1.7×10^8 M^{-1} at 37°C and keq = 6.7×10^8 M^{-1} at 4°C). Another complication in the internalization studies, as previously described by Hand *et al.* (40), is the relatively low level of TAG-72 antigen expression in tumor cell lines grown *in vitro versus* the same tumor cells grown as a nude mouse xenograft. Our data (Figs. 1 and 10) are consistent with the observations of Hand *et al.* (40) and indicate that the TAG-72 antigen is expressed on a low percentage (<5%) of the LS174T and OVCAR-3 tumor cells used in the internalization assays. Caution should therefore be exercised in extending the observations obtained for a small percentage of TAG-72-positive cells in an *in vitro* internalization assay to events which take place *in vivo* with a much higher expression of the TAG-72 antigen. It should be noted, however, that both the LS174T and OVCAR-3 cell lines demonstrated similar characteristics in the B72.3 internalization studies (Figs. 2 to 4), which suggest that the data obtained from these experiments are not merely an artifact unique to a particular cell line. In any event, the lack of internalization activity shown in Fig. 4 and the ability of B72.3 to remain at the tumor site for long periods of time in preclinical and clinical imaging studies (24, 25) suggested that B72.3 forms a stable complex with the TAG-72 antigen that is not rapidly modulated by the tumor cells. In spite of the lack of demonstrated internalization into the tumor cells following antigen binding, B72.3 was used to construct an active immunoconjugate when coupled with DAVLBHYD. Figs. 6 to 9 indicated that B72.3-DAVLBHYD possessed potent antitumor activity *in vivo* against LS174T human colorectal carcinoma xenografts. B72.3-DAVLBHYD was superior to all other treatments (irrelevant conjugate, MoAb plus drug combination, or free drug) for at least one of the doses tested in each experiment (Figs. 6 to 9). Transient regressions were also observed at the 2-mg/kg treatment level (*Vinca* content) of B72.3-DAVLBHYD in 7-day established LS174T xenografts receiving 3, 4, or 6 doses of conjugate (Figs. 7, 8, and 9, respectively). The ability of B72.3-DAVLBHYD to be an effective immunoconjugate in the absence of internalization may be due to the labile nature of the bond used to couple the *Vinca* to the murine IgG (18), which may result in the delivery of free drug to the tumor cell periphery following localization *in vivo* (17). It is clear from the established tumor data shown in Figs. 7 to 9, however, that tumor regrowth is evident at the cessation of therapy of B72.3-DAVLBHYD. Since the TAG-72 antigen is heterogeneously expressed on LS174T tumor cells (Fig. 1; Ref. 34), tumor regrowth may have been due to the expansion of antigen-negative cells that were not killed by the MoAb-drug conjugate treatment. The data in Fig. 10*A*, however, clearly show that heterogeneous TAG-72 antigen expression is present in LS174T tumors even after 6 i.v. doses with 2 mg/kg (*Vinca*) of B72.3-DAVLBHYD. These observations suggest that the problem of antigen heterogeneity may not necessarily be circumvented by the formulation of immunoconjugate combinations which will react with a higher percentage of tumor cells. This prediction may of course be restricted to a particular combination of antibody, linker, and/or drug. For example, a recent report by Engert *et al.* (41) demonstrated that *in vivo* immunotoxin therapy of human Hodgkin's disease xenografts could result in tumor regrowth which was due to the selection of antigen-deficient tumor cells. Data also show that no significant variation in antigen presentation existed in tumor xenografts treated with B72.3-DAVLBHYD (Fig. 10, *A* to *C*) irrelevant IgG1-DAVLBHYD (Fig. 10*F*), or free DAVLBHYD (Fig. 10*E*). It should be noted that the tumors used in this experiment were removed from the animals 15 days following the last conjugate or drug treatment. It is possible that reexpression of TAG-72 antigen occurred during this time interval in the absence of selective pressure. Nevertheless, our data do indicate that the high doses of B72.3-DAVLBHYD used in this study were not sufficient to eradicate all TAG-72 antigen-positive cells from the LS174T tumor xenograft. Our findings are consistent with those of Schlom *et al.* (34) who demonstrated that LS174T xenografts which escaped radiotherapy of ^{131}I-B72.3 had the same antigenic phenotype as untreated tumors.

In conclusion, we have demonstrated that efficacious antitumor chemoimmunoconjugates can be constructed against a target antigen that is heterogeneously expressed and is not rapidly internalized following antibody binding. Such antigen systems represent stringent obstacles for successful solid tumor therapy by MoAb-drug conjugates but may not represent an insurmountable barrier as was previously thought.

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