Secondary mAb−vcMMAE Conjugates Are Highly Sensitive Reporters of Antibody Internalization via the Lysosome Pathway

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Monoclonal antibodies (mAb) selectively recognizing tumor surface antigens are an important and evolving approach to targeted cancer therapy. One application of therapeutic mAbs is drug targeting via mAb−drug conjugate (ADC) technology. Identification of mAbs capable of internalizing following antigen binding has been accomplished by tracking decline of surface-bound mAb or by internalization of a secondary mAb linked to a toxin. These methods may not be sufficiently sensitive for screening nor wholly predictive of the mAbs' capacity for a specific drug delivery. We have developed a highly selective and sensitive method to detect mAbs for cell internalization and drug delivery. This system uses secondary anti-human or anti-murine mAbs conjugated to the high-potency drug monomethyl auristatin E (MMAE) via a highly stable, enzymatically cleavable linker. Prior studies of this drug linker technology demonstrated internalization of a primary ADC leads to trafficking to lysosomes, drug release by lysosomal cathepsin B, and ensuing cell death. A secondary antibody−drug conjugate (2°ADC) capable of binding primary mAbs bound to the surface of antigen-positive cells has comparable drug delivery capability. The system is sufficiently sensitive to detect internalizing mAbs in nonclonal hybridoma supernatants and is predictive of the activity of subsequently produced primary ADC. Because of their high extracellular stability, the noninternalized 2°ADC are 100−1000-fold less toxic to cells over extended periods of time, permitting an assay in which components can be added without need for separate wash steps. This homogeneous screening system is amenable to medium-throughput screening applications and enables the early identification of mAbs capable of intracellular trafficking for drug delivery and release.

INTRODUCTION

Monoclonal antibodies (mAbs) that are selective for tumor cells but not efficacious in antitumor treatment are being increasingly applied as vectors for the therapeutic delivery of toxins or cytotoxic agents to the interior of tumor cells (*1*). Success in this latter application requires that the antibody−drug conjugate (ADC) remain stable in circulation, display tumor selectivity, and be internalized upon binding of surface antigen into an appropriate intracellular pathway facilitating drug release and delivery. The utility of mAbs conjugated to small, highly potent cytotoxics has increased with the advance of more potent agents and development of conditionally labile linkage systems. These linkers exhibit a high degree of stability in plasma and selective drug release within the tumor cell (*2*). Current methods to identify mAbs for drug delivery applications depend on a primary screen for selective binding to the tumor cell, and then on secondary assessment for internalization, prior to construction and evaluation of direct mAb−drug conjugates. Approaches to assessing candidate mAbs for internalization include direct labeling with indicators such as colloidal gold or fluorophores for microscopy (*3*, *4*), direct labeling of mAbs with toxins such as Pseudomonas exotoxin A (*5*), or by use of a secondary mAb, reactive with the first and linked to a toxin such as ricin A chain (*6*). The latter approach, while advancing the field of antibody−drug conjugate discovery, may be skewed by the behavior of the protein toxin compared to the trafficking and release of a high potency small molecule. Additionally, the composition of these toxin conjugates may be heterogeneous and the linkage used in such reporters is not sufficiently stable (*7*) to permit continuous cell exposure without significant background toxicity. Taken together, existing tools may demonstrate characteristics and selectivity that may not be optimal for homogeneous screening of hybridoma arrays for mAb with drug delivery capability.

To speed the discovery of tumor-selective internalizing mAbs from hybridoma screening efforts, we have constructed homogeneous assays employing secondary antibody−drug conjugates (2°ADC), composed of secondary antibodies stably linked to the potent cytotoxic agent MMAE via a valine-citrulline peptide linker (*2*). This linker was originally identified based on its high stability outside of cells and ability to be efficiently and selectively cleaved by lysosomal cathepsin B (*8*). Antigen-dependent internalization and lysosomal release was initially shown for doxorubicin (*8*, *9*), but subsequent studies with taxanes and camptothecin (*10*, *11*) and most recently auristatins (*2*) have extended the capability of this linker to other drugs. Two secondary mAbs were examined with specificities toward IgGs of either human or mouse origin and exhibited cytotoxicity dependent upon the binding, internalization, and lysosomal transit characteristics of the primary antibody. We have previously shown the drug remains stably appended to the mAb in serum or plasma and is highly attenuated when incubated with

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antigen-negative cells (*2*, *12*). Upon ADC binding and internalization in antigen-positive cells, the drug linkage is fragmented by lysosomal proteases (*8*) releasing the cytotoxic agent MMAE into the cytosol. Internalization results in a 100−1000× increase in selectivity over noninternalizing mAb−2°ADC pairs. Thus the system is sufficiently sensitive to identify small amounts of internalizing mAbs in heterogeneous mixtures such as nonclonal hybridoma cultures.

MATERIALS AND METHODS

Cells and Reagents. Hybridomas for mAb HB43, a murine IgG1 reactive with human IgG1 gamma heavy chain (*13*), mAb 187.1, a rat IgG1 reactive with mouse kappa light chain (*14*), and OKT9, a murine IgG1 specific for human transferrin receptor (*15*), were obtained from the American Type Culture Collection (ATCC, Manassas, VA). mBR96 is a murine IgG3 reactive with an extended form of the Lewis Y (Le^y) antigen found on numerous human carcinoma cells (*16*). cBR96 is a chimeric form of mBR96 composed of a human IgG1 H and L framework (*17*). mAC10 is a murine IgG2b reactive with human CD30 (18). cAC10 is a chimeric form of mAC10 composed of a human IgG1 H and L framework (*19*). mAb hup97 is a human IgG1 directed against melanotransferrin. cL6 is a chimeric form of mL6, a monoclonal antibody recognizing a tumor-associated antigen, known as L6 antigen, present on carcinomas of the lung, breast, colon and ovary, composed of a human IgG1 H and L framework (20). H460, H358, H226, and A549 lung carcinoma and MDA-MB-453 breast carcinoma lines were obtained from ATCC. H3677 and H3396 breast carcinoma were gifts from Dr. I. Hellstrom (Pacific Northwest Research Foundation, Seattle, WA). LX-1 lung carcinoma was a gift from Dr. E. Neuwelt (Oregon Health Sciences University, Portland, OR) and was originally isolated at the Mason Research Institute, Worcester, MA. The anaplastic large cell lymphoma (ALCL) line, Karpas 299, was obtained from the DSMZ (Braunschweig, Germany). All cell lines and hybridomas were grown in RPMI-1640 media (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies Inc.) and were verified to be mycoplasma-free by PCR (ATCC Mycoplasma detection kit, American Type Culture Collection, Manassas, VA). Antibody was purified from culture supernatants by Gamma Bind (Amersham Pharmacia Biotech, Piscataway, NJ) chromatography. Goat anti-mouse-FITC and goat anti-human-FITC were from Jackson Immunoresearch, (West Grove, PA).

Linker Synthesis. The synthesis of the activated valcit linkage system to append drug was modified from the previously described procedure (*9*).

Drug Synthesis. The synthesis of auristatin E has been previously described (*21*). The monomethyl derivative of auristatin E (MMAE) was prepared by replacing a protected form of monomethylvaline for *N*,*N*-dimethylvaline in the synthesis of auristatin E. MMAE was then further modified with an activated derivative of maleimidocaproyl-valine-citrulline that contained a *p*-aminobenzylcarbamate spacer between the MMAE and the linker as previously described (*2*).

Conjugate Preparation. *Antibody Reduction.* To 5 mL of each mAb (10 mg/mL) was added 600 μL of 500 mM sodium borate/500 mM NaCl, pH 8.0, followed by 600 μL of 100 mM DTT in water. After incubation at 37 °C for 30 min, the buffer was exchanged by elution over G25 resin equilibrated and eluted with PBS containing 1 mM DTPA (Aldrich, Milwaukee, WI). The thiol/Ab value was checked by determining the reduced antibody concentration from the absorbance at 280 nm of the solution, and the thiol concentration by reaction with DTNB (Aldrich) and determination of the absorbance at 412 nm.

Conjugation of the Reduced Antibody. The reduced mAb was chilled on ice. The drug-linker (vcMMAE) was used as a frozen DMSO solution of known concentration, and the quantity of drug-linker added to the reaction mixture was calculated as follows:

L stock solution = $V \times$ [Ab] × fold excess/[drug-linker], where V and [Ab] are the volume and molar concentration of the reduced antibody solution, respectively. Cold PBS/DTPA (2.3 mL) was added to each of the two reduced antibody solutions. For the vcMMAE conjugate, 133.6 μL of 7.5 mM maleimidocaproyl-vcMMAE stock solution was diluted into 1.47 mL of acetonitrile. The acetonitrile drug-linker solution was chilled on ice then added to the reduced antibody solutions. The reactions were terminated after 1 h by the addition of a 20-fold molar excess of cysteine over maleimide. The reaction mixtures were concentrated by centrifugal ultrafiltration and purified by elution through desalting G25 in PBS. ADCs were then filtered through 0.2 μm filters under sterile conditions and immediately frozen at −80 °C. ADCs were analyzed for concentration, by UV absorbance; aggregation, by size exclusion chromatography; drug/Ab, by measuring unreacted thiols with DTNB; and residual free drug, by reverse phase HPLC. The procedure reproducibly yielded ADC of <3% aggregate, 8 drugs/mAb and <1% free drug.

Flow Cytometric Analysis of mAb Binding. To evaluate binding of the primary mAbs to cells, 4×10^5 cells were combined with mAb at 10 μg/mL in ice-cold staining media (PBS/2% FBS/0.02% sodium azide) for 30 min on ice and washed twice with ice cold PBS. Cells were then incubated with either goat anti-mouse-IgG FITC or goat anti-human-IgG-FITC, depending on the primary mAb at 10 μg/mL in ice-cold staining media on ice for 30 min and washed as described above. Labeled cells were resuspended in 0.3 mL of PBS and then analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, Mansfield, MA) using the CellQuest software package. Data are shown in histogram format with fluorescence intensity displayed in log intervals on the *x*-axis and number of events (linear scale) on the *y*-axis.

Cytotoxicity Assays. Cell lines were plated at previously determined densities (based on proliferation rates and Alamar Blue labeling efficiency) in 100 μL of complete media and cultured overnight to adhere to the well. Primary mAb and 2°ADC were combined at a ratio of 1:2 in complete media and allowed to complex at room temperature for 15 min prior to serial dilution in complete media. Fifty microliters of these dilutions was added to the cell plate, and the plates were incubated at 37 °C for 96 h. At 92 h cells were labeled with Alamar Blue (Biosource International, Camarillo, CA), at a final concentration of 10% culture volume, and cytotoxicity was measured according to manufacturers directions on a Fusion HT fluorescent plate reader (Packard Instruments, Meriden, CT). (*22*). Raw data was analyzed in an Excel spreadsheet and cytotoxicity expressed by dose−response curves plotted for percent cell viability compared to untreated control wells (*y*-axis), versus concentration of mAb/ADC treatments (*x*-axis).

For comparison of the activity of the 2°ADC with two commercially available secondary immunotoxins (2° IT), Mab-ZAP (*23*) and Hum-ZAP (Advanced Targeting Systems, San Diego, CA), MDA-MB-453 breast carcinoma

cells (Le^{y+}) were plated at 1×10^5 cells/mL (0.1 mL/well) in RPMI 1640 + 10% FBS and allowed to incubate overnight at 37 °C. The following day the 2°ADC or 2°IT was added to cultures at 2 μg/mL (100 ng/well) in complete medium. Immunotoxins consisted of goat-anti-mouse IgG-saporin (Mab-ZAP) or a goat-anti-human-saporin (Hum-ZAP). Serial dilutions of mAb were then added to the cultures and incubated for 96 h. Cytotoxicity was measured by reduction of Alamar Blue. Secondary reagents were tested in the absence of targeting mAb in this system and had no effect on cell growth.

Evaluation of Hybridoma Supernatants and Purified mAb for Internalization by 2°ADC. Three NSCLC cell lines, H460, H358, and H226, were combined and plated at 1×10^3 cells of each line/well in complete media and allowed to adhere overnight. As released drug is distributive to bystander cells, combining multiple cell lines allows identification of internalization if only one cell line is antigen positive. Fifty microliters of neat hybridoma-conditioned culture supernantant from a NSCLC immunization/fusion was combined with 2°ADC, allowed to complex for 15 min at RT, then added to cells for a 6-day incubation at 37 °C. Master wells were frozen for subsequent reculture. Alamar Blue label was added for the last 4 h of culture and fluorescence measured to assess cell viability compared to cells in untreated control wells. Frozen master wells were recultured, and those with supernatant showing >60% cytotoxicity were recloned and the conditioned media evaluated as above. This evaluation was continued through subsequent rounds to purified antibody from isolated clonal cells.

RESULTS

Characterization of mAbs and Cell Lines. As models for evaluation, we selected mAbs and antigen expressing cell lines previously shown to have varying levels of internalization following mAb binding. Cell lines used were also demonstrated to show similar sensitivity to MMAE, with IC_{50} values of the following: 0.88 nM, A549; 0.07 nM, Karpas 299; 0.4 nM, H3396; 1.2 nM, LX-1; and 0.52 nM, H3677. mAb BR96 targets an extended form of the Le^y antigen present on numerous carcinomas and undergoes rapid internalization following antigen ligation (*16*). mAb OKT9 recognizes the transferrin receptor (CD71) expressed on most dividing cells and has also been shown to undergo efficient internalization upon antigen binding (*24*). We have recently shown that cAC10, a chimeric antibody against the CD30 antigen present on numerous hematalogic malignancies, is a highly effective internalizing mAb for intracellular drug delivery (*12*). Murine AC10 and cAC10, a chimeric version of AC10, were evaluated both as an unmodified mAb in combination with 2°ADC, and cAC10 as a direct antibody−drug conjugate. mAb L6 is reactive with L6 antigen expressed on lung, breast, and colon carcinomas (*25*). mAb L6 has been shown to mediate both complement activation (CDC) and antibody-dependent cellular cytotoxicity (ADCC) leading to the destruction of human tumor cells in vitro (*26*), yet was shown to be poorly internalizing when examined as a conjugate with ricin A chain toxin (*27*). Cytotoxicity, and presumably internalization of mAb L6-toxin conjugates, could be increased in combination with the lysosomal disrupting agent listeriolysin, suggesting that internalization of L6-toxin was not followed by release of the active toxin from the lysosome (*27*) and further suggesting that mAb L6 could not effectively deliver a drug via the lysosomal pathway. mAbs hup97 and L49 bind the human melanoma-associated antigen, melanotransferrin (p97). P97 is stably expressed on the cell surface and not detectably internalized upon mAb binding (*28*, *29*). The p97 mAb/Ag complex served as a noninternalizing control in these studies. The antigen expressing lines, reactive antibodies, and respective 2°ADC are summarized in Table 1.

Table 1. Primary and Secondary Reagents

antigen	primary mAb	secondary ADC
CD71	OKT9	187.1-vcMMAE (RAM-MMAE)
Le^Y	cBR96	HB43-vcMMAE (MAH-MMAE)
Le^Y	mBR96	187.1-vcMMAE (RAM-MMAE)
L6 Ag	cL6	HB43-vcMMAE (MAH-MMAE)
p97	huAnti-p97	HB43-vcMMAE (MAH-MMAE)
CD30	cAC10	HB43-vcMMAE (MAH-MMAE)
CD30	mAC10	187.1-vcMMAE (RAM-MMAE)

Comparison of p97- and L6-Targeted Internalization. To evaluate the 2°ADC reporter, H3677 melanoma cells and A549 and LX-1 lung carcinoma cell lines were evaluated for cytotoxicity in response to internalization and drug release by mAb/2°ADC complexes. Figure 1A shows FACS analysis of the relative levels of binding of the previously reportedly noninternalizing mAbs L6 and L49 to their respective antigens on A549, LX-1, and H3677 cells as compared to binding of the secondary FITC reporter reagent alone. FACS evaluations were done at 4 °C in the presence of NaN_3 to stabilize surface binding and to block modulation. All three cell lines were positive for both antigens, with the L6 antigen showing ~1-log greater expression as judged by mAb binding compared to p97.

The cytotoxicity of L6-positive, p97-positive cells was examined in response to mAbs, 2°ADCs, or mAb/2°ADC complexes. Figure 1B shows the sensitivity of LX-1 cells to mAb L6/2°ADC or mAb p97/2°ADC complexes or the 2°ADCs alone. mAb L6/2°ADC was significantly more potent (IC_{50} = 1 μg/mL) than either mAb p97/2°ADC complexes or the 2°ADC alone (IC_{50} indeterminate). Figures 1C and 1D show parallel studies preformed on A549 (lung) and H3677 (melanoma) lines, respectively. As with LX-1 cells, only the combination of anti-L6 and 2°ADC resulted in significant cytotoxicity (IC_{50} on A549 and H3677 = 9 ng/mL and 10 ng/mL, respectively) while nominal cytotoxicity was observed from treatment with the noninternalizing anti-p97/2°ADC complexes or the 2°ADC alone (IC_{50} > 5 μg/mL). Together these data indicate the primary mAb L6 was efficiently internalized to deliver the associated 2°ADC with cytotoxic effect and, contrary to early reports examining L6-toxin conjugates (27), suggest mAb L6 provided efficient delivery of a small, high potent drug.

Le^y-, CD30-, and CD71-Targeted Internalization. To evaluate internalization of mAbs directed to antigens Le^y, CD30 and CD71, the breast carcinoma line H3396 and the anaplastic large cell lymphoma line Karpas 299 were first analyzed for the presence of these antigens by flow cytometry. Figure 2A (upper panels) shows that H3396 cells stained strongly with cBR96 (against the Le^y antigen) moderately with OKT9 (CD71 antigen) and not with cAC10 (CD30 antigen). Karpas 299 cells displayed moderate to high levels of staining with both cAC10 and OKT9, reflecting high expression of the CD30 and CD71 antigens respectively, and nominal staining for Le^y (Figure 2A, lower panel). Together, H3396 and Karpas 299 cells provided reciprocal, nonbinding controls for these studies. As both cell lines expressed CD71, mAb OKT9 was used as a common positive control for internalization.

Using the CD30+ cell line, Karpas 299, both AC10 and cAC10 forms of the CD30 antibody were evaluated for

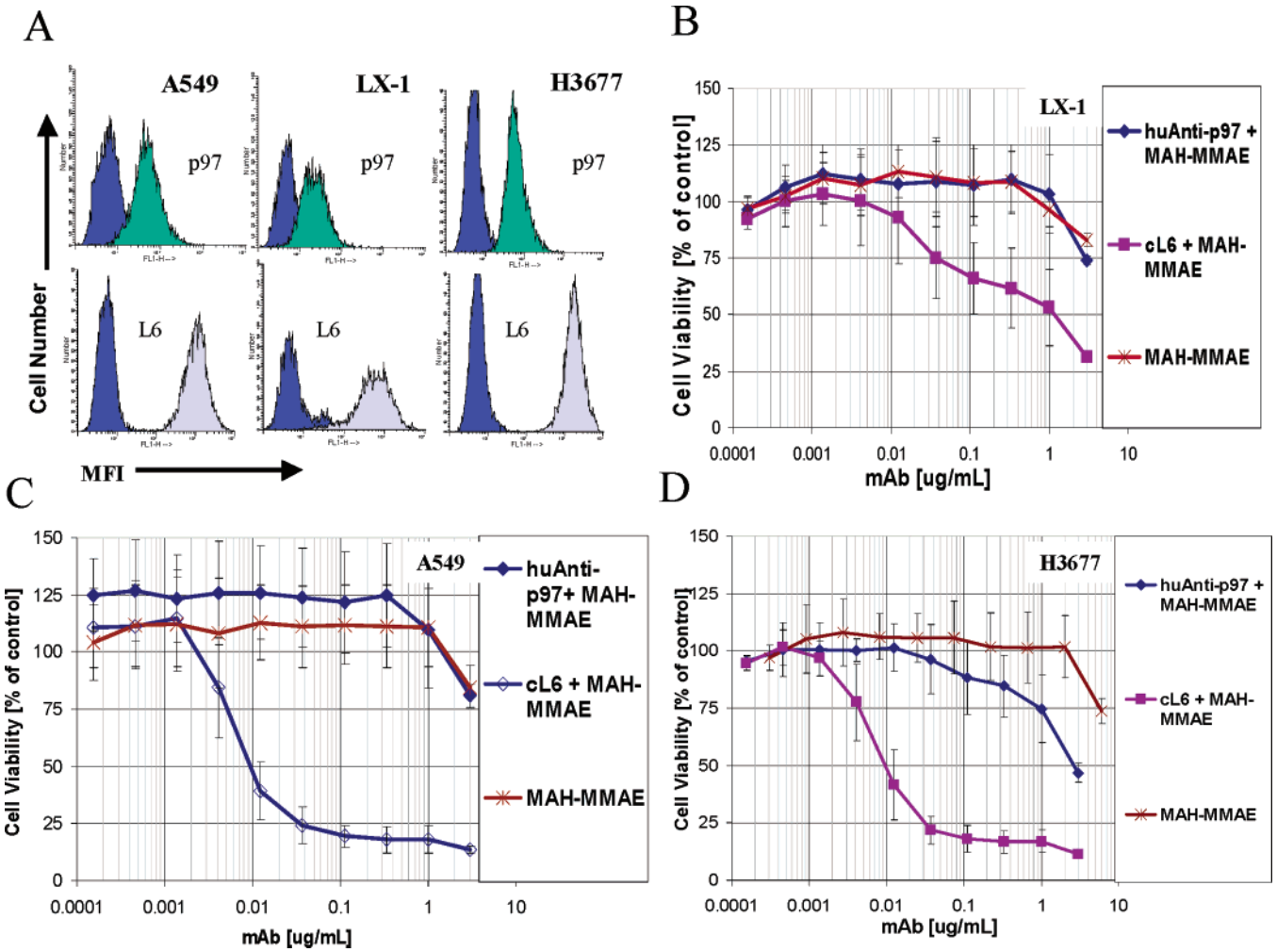

Figure 1. (A) Evaluation of p97 and L6 mAbs on tumor cells. A549, LX-1, and H3677 tumor cells (4 × 10^5 of each) were stained for 30 min on ice with 10 μg/mL of the designated primary mAbs, washed as described in Materials and Methods followed by incubation with 10 μg/mL FITC-labeled secondary Ab for 30 min on ice. After final washes, cells were resuspended in PBS and the resultant fluorescent intensity analyzed by flow cytometry. Blue histograms indicate staining with secondary anti-human FITC only. Shaded histograms, top panel huAnti-p97/secondary anti-human FITC, bottom panel cL6/secondary anti-human FITC. (B) Sensitivity of LX-1 cells to a secondary 2°ADC complexed with mAb-p97 or mAb-L6. (C) Sensitivity of A549 cells to a secondary 2°ADC complexed with mAb-p97 or mAb-L6. (D) Sensitivity of H3677 cells to a secondary ADC complexed with anti-p97 or anti-L6. Cells (2 × 10^3) were plated in microtiter wells in complete media and allowed to adhere overnight, and titrations of primary mAbs complexed with a 2°ADC were added. Mab/2°ADC complexes were preformed at RT for 15 min, and titrations were made in complete media and added to plates containing cells. Cultures were incubated for a total of 96 h at 37 °C, and Alamar Blue label was added for the last 4 h of incubation. A 96-well fluorescence plate reader measuring emission from metabolized dye was used to assess cell viability compared to control wells containing cells as described in Materials and Methods. Shown are the dose−response curves resulting from primary mAb−2°ADC complexes and 2°ADC alone. Data points are the mean of quadruplicate determinations.

CD30-targeted internalization using corresponding anti-mouse or anti-human 2°ADCs (Figure 2B). The nonbinding and binding primary mAbs mBR96 and OKT9 were included as negative and positive controls, respectively, as were the two 2°ADCs alone. Treatment with either AC10 or cAC10 in combination with their respective 2°ADCs resulted in similar IC_{50}s of 3−4 ng/mL. OKT9/2°ADC complexes gave intermediate toxicity (IC_{50} = 50 ng/mL), perhaps attributable to the lower CD71 levels compared to CD30 on the cells (Figure 2A). In contrast, neither the nonbinding control complex, BR96/2°ADC, nor the 2°ADCs alone resulted in cytotoxicity at concentrations below 3 μg/mL (Figure 2B). These results suggest that the 2°ADCs were capable of internalization and delivering drug to the cytosol only when complexed with the internalizing anti-CD30 mAbs or anti-CD71 mAbs. The ∼1000-fold potency difference between relevant and irrelevant mAbs also suggest the 2°ADCs remain intact and do not release free drug outside the cell during the course of the 96 h assay.

In a reciprocal experiment, Le^y-positive/CD30-negative H3396 cells were treated with mBR96 or cBR96 (binding Le^y) and their appropriate anti-mouse or anti-human 2°ADCs. mAb OKT9, binding CD71 on these cells, and the nonbinding mAb mAC10 were also evaluated in the presence of 2°ADCs, as were the two 2°ADCs alone. Figure 2C shows moderate H3396 sensitivity to OKT9/2°ADC and high sensitivity to the two BR96 mAbs in the presence of their respective 2°ADCs. IC_{50}s were 80 ng/mL for OKT9/2°ADC and 20−25 ng/mL for the cBR96/2°ADC and mBR96/2°ADC, respectively. The difference in sensitivity may be attributable, in part, to the ∼2-logs greater expression of Le^y compared to CD71 on H3396 cells. IC_{50}s for the 2°ADCs alone were indeterminate, and no toxicity was observed with the primary mAbs alone tested at up to 3 μg/mL (data not shown).

Comparison of mAb/2°ADC Complex versus Primary ADC. The results of mAb/2°ADC studies shown above suggested that a direct drug conjugate constructed from mAbs cAC10 or cL6 would efficiently kill respective

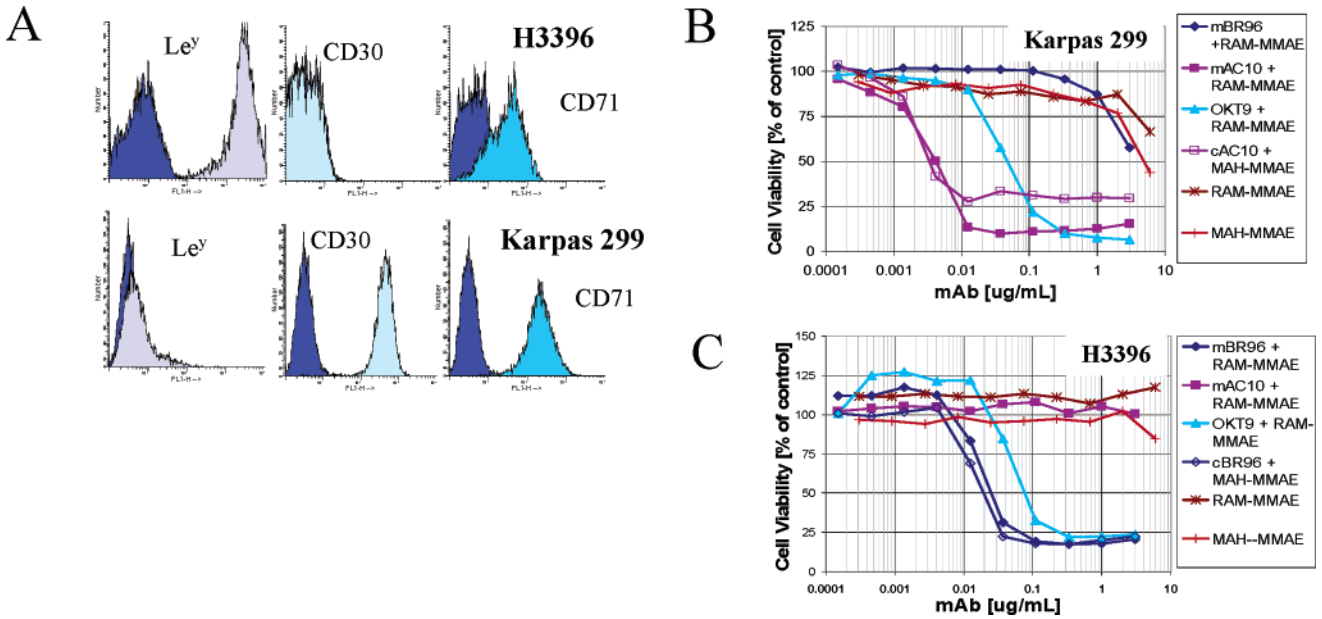

Figure 2. (A) Binding levels of mAb to Le^y, CD30, and CD71 on H3396 and Karpas 299 cells. Tumor cells (4×10^5) were pelleted and resuspended in 10 μg/mL primary mAb in ice-cold staining media and incubated on ice for 30 min, washed as described in Materails and Methods, and resuspended in 10 μg/mL FITC-labeled secondary Ab in ice-cold staining media. Following a 30-min incubation on ice, cells were washed, resuspended, and analyzed by flow cytometry as described in Materials and Methods. Blue histograms indicate FITC-labeled secondary antibody staining only, anti-human FITC (left and center panels), and anti-mouse FITC (right panel). Shaded histograms indicate staining in conjunction with primary mAbs, cBR96 (left panel), cAC10 (center panel) and OKT9 (right panel). (B). Cytotoxic effect of chimeric and murine anti-CD30 mAbs combined with respective 2°ADCs on CD30-positive Karpas 299 cells. Karpas cells were plated at 1×10^4/well in 100 μL of complete media. Precomplexed primary mAbs + 2°ADCs were titrated and added to wells containing cells. Cultures were incubated 96 h at 37 °C with addition of Alamar Blue label for the last 4 h of culture. Fluorescence from the metabolized dye was measured to assess cell viability compared to control wells containing untreated cells as described in Materials and Methods. Response curves show the effects of primary mAbs cAC10 (chimeric) and mAC10 (murine), complexed with their appropriate 2°ADC. IC_{50} values in μg/mL = 0.0034 and 0.004, respectively. Response curves for irrelevant control mAb mBR96/2°ADC complex, IC_{50} = >3 μg/mL, positive internalization control mAb OKT9/2°ADC complex, IC_{50} = 0.047 μg/mL, and the secondary ADCs alone, with IC_{50} values ≥ 5 μg/mL are also shown. C. Cytotoxic effect of chimeric and murine anti-Le^y mAb BR96 combined with anti-human or anti-mouse 2°ADCs on Le^y-positive H3396 carcinoma cells. Cells were plated at 4×10^3/well in microtiter wells in 100 μL/well complete media. Following overnight adherence, titrations of primary mAb + 2°ADC that had been allowed to precomplex for 15 min at RT were added in 50 μL/well, and the assay evaluation was carried out as in B above. Response curves indicate effects of primary mAbs mBR96 (murine) and cBR96 (chimeric), complexed with their appropriate 2°ADC. IC_{50} values in μg/mL = 0.024 and 0.02, respectively. Also shown are response curves for positive internalization control mAb OKT9/2°ADC complex, IC_{50} = 0.075 μg/mL, irrelevant control mAb mAC10/2°ADC complex, and the secondary ADCs alone, IC_{50}s indeterminate. Data points are the mean of quadruplicate determinations.

antigen-positive cells. We have previously shown that conjugation of mAbs via this method does not change binding characteristics as compared to the parent mAb (*2*, *12*). Primary ADCs were prepared from the two mAbs, and their potency and selectivity were compared with that of the primary mAb/2°ADC complex.

The L6-positive cell line A549 was used to evaluate mAb cL6 in the form of a primary ADC (cL6−vcMMAE) or L6 mAb/2°ADC complex, and the resultant dose−responses are shown in Figure 3A. The primary ADC and the primary mAb/2°ADC complexes showed comparable cytotoxicity (IC_{50} = 0.02−0.03 μg/mL for either treatment) whereas the 2°ADC alone had no significant effect.

In a parallel study, the cytotoxicity of a direct conjugate of mAb cAC10 (cAC10−vcMMAE) was compared with that of a cAC10 mAb/2°ADC complex (cAC10 + MAH−vcMMAE). Figure 3B shows CD30-positive Karpas 299 cells treated with either cAC10 + MAH−vcMMAE or cAC10−vcMMAE resulted in IC_{50}s = 8 and 4 ng/mL, respectively. These data suggest that cytotoxicity resulting from the screening with primary mAb/2°ADC complexes would be predictive of the potency of a subsequently produced primary ADC. In the absence of a target-specific primary mAb, the 2°ADC alone demonstrated limited or no cytotoxicity with an IC_{50} not reached for the 2°ADC alone on cells treated up to 6 μg/mL.

Comparison of 2°ADC with 2°IT. The Le^y-positive/CD30-negative cell line MDA-MB-453 was used to compare the potency of a secondary vcMMAE ADC with that of a commercially available 2° IT. Using either mBR96 or cBR96 in combination with their respective 2°ADC gave IC_{50}s of 0.12 and 0.3 μg/mL (Figure 4). In contrast, the IC_{50} was indeterminate using a secondary anti-human immunotoxin (Figure 4A). The potency of an anti-mouse immunotoxin to detect internalization of the mBR96 was diminished 100-fold over that detected with a secondary anti-mouse ADC (Figure 4B). No cytotoxicity was detected with either secondary reagent alone or these secondary reagents in the presence of an irrelevant (anti-CD30) primary mAb at exposures up to 2 μg/mL (data not shown). These data suggest that the route of internalization and degradation of BR96 mAbs is more amenable to delivering the vcMMAE ADC than a protein toxin, as only the 2°ADC reagents retained their antigen selectivity and drug delivery capability in the presence of primary mAb over the concentration range examined.

Screening of Hybridoma for Internalizing mAb. The characteristics of the secondary anti-mouse ADC, high stability and attenuated toxicity outside of cells and highly potent cytotoxicity upon internalization, suggested it could be used to identify internalizing mAbs at low concentration early in the hybridoma screening process. To examine this utility, a primary hybridoma fusion,

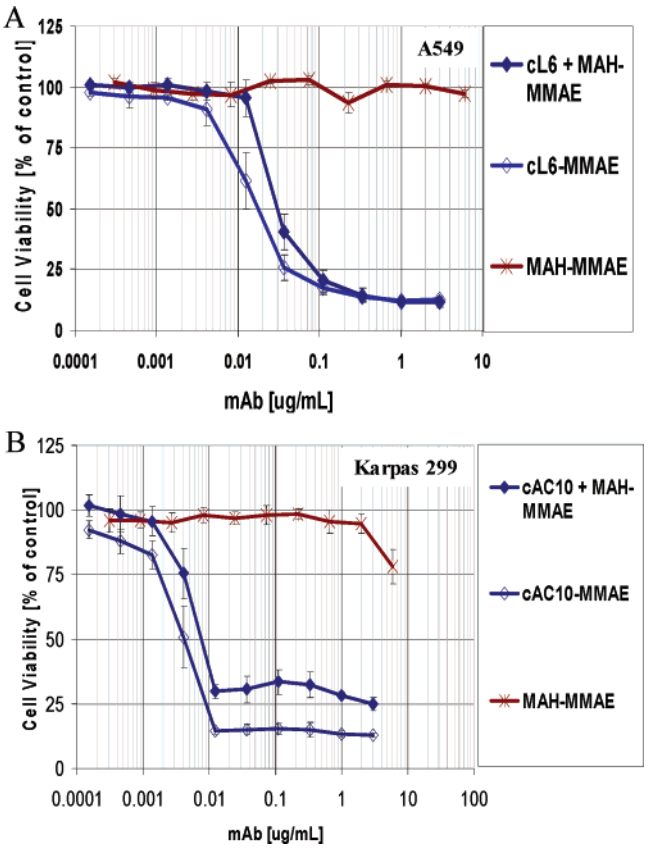

Figure 3. Comparative cytoxicity of primary ADC versus primary mAb/2°ADC complex. (A) mAb cL6 on L6 antigen-positive cells. A549 carcinoma cells were plated at 1×10^3/microtiter well in 100 μL/well complete media. Following overnight adherence, cells were exposed to a titration of either a primary antibody−drug conjugate or a primary antibody complexed with a 2°ADC. Mab/2°ADC complexes were preformed at RT for 15 min, and titrations were made in complete media and added to plates containing cells. Cultures were incubated for a total of 96 h at 37 °C, and Alamar Blue label was added for the last 4 h of incubation. A 96-well fluorescence plate reader measuring emission from metabolized dye was used to assess cell viability compared to control wells containing cells as described in Materials and Methods. Dose−responses shown are for primary mAb/2°ADC complexes, primary ADC, and 2°ADC alone. All data points are an average of quadruplicate determinations. (B) anti-CD30 on CD30-positive cells. CD30 positive Karpas 299 cells were plated at 1×10^4/well in 100 μL of complete media. Titrations of either a primary ADC or primary mAb/2°ADC complex were added to cells plated in microtiter wells and assays carried out as in A above. Data points are the mean of quadruplicate determinations (±SE).

producing mAb against an immunization of multiple NSCLC membrane antigens was arrayed in 15 96-well plates at ∼4−10 different clones/well. The hybridoma were cultured for 5 days followed by three daily media changes. Following this, hybridoma were allowed to condition the media for an additional 72 h, and an aliquot of culture supernantant was removed for screening. Hybridoma plates were frozen for subsequent reculture, and an aliquot of supernatant (50 μL) was combined with a final concentration of 2 μg/mL of secondary anti-mouse ADC and added to wells containing normal lung epithelium or the NSCLC line mixture H460, H358, and H226. Free MMAE is distributive, and ADC internalization and drug release by one antigen-positive cell will result in cytotoxicity of neighboring antigen-negative cells (data not shown). Cultures were incubated for 6 days to allow maximum opportunity of internalization and resultant toxicity evaluated as described above. This method identified multiple wells in which viability was reduced by >60% compared to untreated control (Figure 5A). Plates were thawed, positive wells were expanded, and the screening was repeated with serial dilution. Figure 5B shows the titrated cytotoxicity of supernatant from clone 5D7 and other positive cultures following secondary expansion of masterwells in combination with 2°ADC. Following reexpansion and final cloning of well 5D7, the mAb was purified and evaluated for cytotoxicity in combination with 2°ADC in parallel with known internalizing and noninternalizing controls (Figure 5C). Of note, mAb 5D7 was identified as an IgM, and its stability as a purified mAb has been problematic. This could account for the diminished activity observed with the purified antibody (Figure 5C) but points up the ability of this system to identify both IgG and IgM isotypes.

These data suggest the 2°ADC is sufficiently sensitive to identify low levels of an internalizing mAb in a small volume of nonclonal hybridoma supernatant.

DISCUSSION

The successful use of antibody−drug conjugates for targeted-tumor drug delivery requires that the ADCs be directed to the site via tumor-selective antigens, undergo internalization through antigen modulation, and release drug by intracellular processes. We have combined the elements of a highly potent cytotoxic agent, MMAE, with a highly stable valine-citrulline linker system that is selectively cleavable by lysosomal proteases (*2*, *9*). This lysosomally cleavable linker has been shown to be broadly applicable to intracellular delivery of a variety of cytotoxic agents including doxorubicin, paclitaxel, and camptothecin (*8*−*11*). The resultant ADCs are potent drug delivery systems to antigen-positive cells yet are significantly less toxic to cells lacking the antigen even under conditions of sustained exposure (*2*, *12*). These qualities also suggested this ADC system could be applied early in the screening process for the identification of mAbs that would optimally internalize and deliver drug.

In the course of discovery of internalizing mAbs it would not be practical to prepare primary drug conjugates from the thousands of mAb candidates emanating from a single hybridoma fusion. A screening strategy for internalizing antibodies using a secondary antibody coupled to a protein toxin was initially described by Till et al. (*6*). We have applied this strategy using a novel drug linker and describe here an approach to the discovery of internalizing mAbs using highly sensitive secondary anti-mouse or anti-human antibody−drug conjugate (2°ADC) reporters. These permit the screening of heterogeneous mixtures at low concentrations typically found in early, nonclonal hybridoma supernatants. In the presence of the 2°ADC, internalizing primary mAbs can be clearly identified by resultant cytotoxicity of cultured tumor cell lines using a homogeneous format. The system provides several appealing features: the high potency of the released drug, MMAE, produces significant cytotoxicity only in the presence of an internalizing primary mAb, whereas the attenuation and high stability of the 2°ADC outside the cell allows them to be combined with test cells in the absence of a internalizing mAb for extended periods of time (observed up to 6 days) without appreciable increase in cytotoxicity. This characteristic also allows for addition of the primary mAb and 2°ADC, and eventually cell viability reagent, without the need for intermediate wash steps. The necessity to wash cells at progressive steps adds significantly to the work and assay variability when assessing thousands of hybridoma

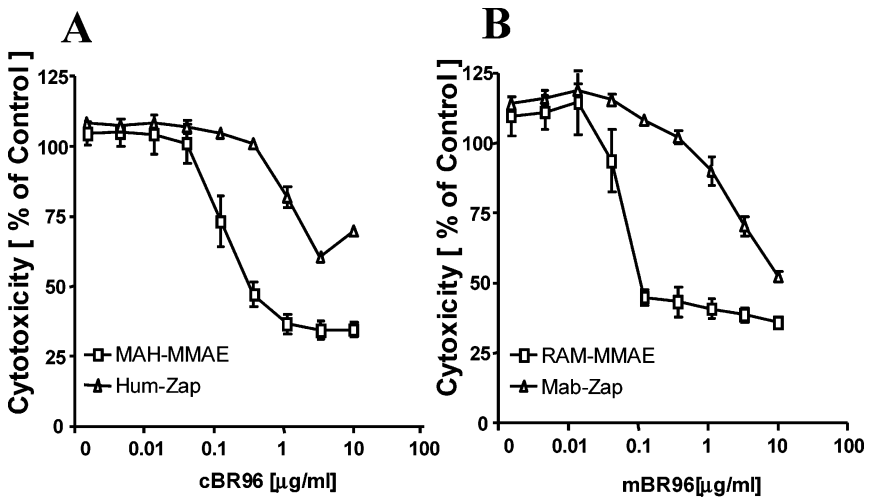

Figure 4. Comparison of 2°ADC and 2°IT reporters. The cell line MDA-MB-453 (Le^y-positive/CD30-negative) was used to compare the potency of a secondary vcMMAE ADC with that of a 2°IT. Cells were exposed to (A) human chimeric mAb BR96 combined with either MAH-MMAE or with Hum-ZAP, or (B) murine mAb BR96 combined with either RAM-MMAE or with Mab-ZAP. Viability was assessed by Alamar Blue metabolism following 96 h incubation. Data points are the mean of quadruplicate determinations (± SE).

supernatants. Additionally, the continuous cell exposure for the duration of a 6 day assay of such supernatants produces >1000-fold difference between internalizing and noninternalizing primary mAb, allowing the identification of even very weakly internalizing mAbs or those present at low (ng/mL) concentrations.

Importantly, comparison of mAb conjugated to drug and immunotoxin (Figure 4) suggests that evaluation of a mAb via a 2° IT reporter may not be predictive of the potential of this mAb to deliver a small drug from conjugate. Previous studies with immunotoxin reporters used primary mAb at 10 μg/mL in order to detect internalization (*16*). In the present study cytotoxicity is clearly detectable at 1/100 this level of primary mAb. It is unclear whether these differences are a function of drug potency or alternate routes of trafficking and release, but worth noting that under the conditions described, the 2° IT would likely not have identified mAb BR96 as internalizing. At the highest concentration of cBR96 tested, cytotoxicity appeared to decrease with the Hum-ZAP (Figure 4A), suggesting that optimal sensitivity is dependent on an optimal ratio of primary mAb and secondary indicator. This was not seen with the 2°ADC reporter suggesting it is less sensitive to the ratio of primary and secondary mAb.

We have examined the effect of 2°ADC reagents on cytotoxicity when used in combination with mAbs and antigens previously reported to rapidly internalize and recycle (*24*), internalize upon mAb ligation (*16, 12*), or not to undergo antigenic modulation after mAb binding (*28, 29*). In general our results with the 2°ADC confirm the previously reported behavior of the primary mAbs. Treatment of the cell lines H3396 and Karpas 299 with anti-transferrin receptor mAb, OKT9/2°ADC, resulted in comparable cytotoxicity on the antigen-positive lines. The internalizing mAb BR96, in both murine and chimeric forms, was cytotoxic on Le^y+ H3396 and diminished by >1000-fold on antigen-negative Karpas 299 cells (Figure 2A,B). Conversely, mAb AC10, tested in either its murine or chimeric form, complexed with 2°ADC, showed potent cytotoxicity on the CD30+ line Karpas 299 and diminished by >1000-fold on antigen-negative H3396. Comparison of the direct conjugate cAC10−vcMMAE and the cAC10/2°ADC complex gave similar cytotoxicity on CD30-positive cells, suggesting that results with the mAb/2°ADC complex can predict the activity of a direct ADC made from the primary mAb. Additionally, the comparable cytotoxicity resulting from murine and chimeric forms of a mAb in combination with anti-mouse or anti-human 2°ADC may be a useful indicator of how well antigen recognition and trafficking is preserved in the chimerization or huminazation process.

Studies with the noninternalizing antigen p97, when evaluated with anti-human p97 mAb/2°ADC on three antigen-positive lines, showed significant cytoxicity only at 2−3 logs higher concentration than other mAbs. These results are in agreement with the previous observation that p97 does not undergo mAb-mediated modulation.

mAb L6 has been shown to mediate both CDC and ADCC activity (26), yet L6 conjugated to ricin A chain internalized poorly upon Ag binding (27). Interestingly, treatment of all three L6 antigen-positive lines with mAb cL6/2°ADC resulted in significant cytotoxicity (Figure 1B−D), an effect paralleled by the potency of the direct cL6−vcMMAE conjugate. These data again suggest that the efficacy of mAb−toxin conjugates or secondary mAb−toxin reporters may not be predictive of the activity of the same mAb delivering a drug such as MMAE, and that the 2°ADC system is predictive of the activity of an ADC subsequently produced with the primary mAb.

The system described herein enables the identification of internalizing mAbs against tumor cell antigens based on their optimal delivery of this drug linker system. Because of the significant (3−4 log) increase in cytotoxicity upon binding and internalization, the system is amenable to the identification of internalizing mAbs in dilute and heterogeneous media from hybridoma culture. The high stability and low toxicity of the 2°ADC linker system outside the cell allow for a simple, homogeneous screening tool with significant signal-to-noise ratio when performed over 96 h of continuous exposure. To this end we have successfully used this method to screen a series of hybridoma-conditioned supernatants to identify tumor reactive, internalizing mAbs. The released MMAE is distributive, and ADC internalization and drug release by one antigen-positive cell will result in cytotoxicity of neighboring antigen-negative cells. Thus multiple carcinoma lines can be combined in screening for internalizing antibodies against unknown tumor markers. One mAb identified in this manner and conjugated to vcMMAE was shown to be a potent and selective antitumor agent (A. F. Wahl, manuscript in preparation), validating the

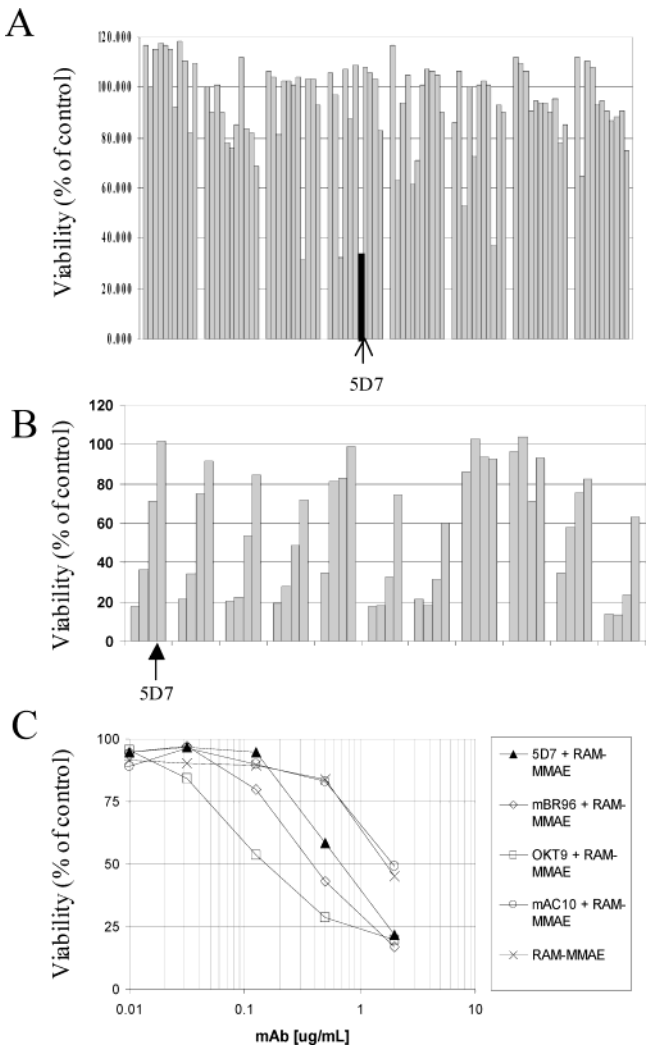

Figure 5. Evaluation of hybridoma supernatants and purified mAb for internalization by 2°ADC. (A). Three NSCLC cell lines, H460, H358, and H226, were combined and plated at 1×10^3 cells of each line/well and allowed to adhere overnight. Fifty microliters of neat hybridoma-conditioned culture supernatant from a NSCLC immunization/fusion were combined with 2°ADC, allowed to complex for 15 min at RT, and then added to cells for a 6-day incubation at 37 °C. Alamar Blue label was added for the last 4 h of culture and fluorescence measured to assess cell viability compared to cells in untreated control wells as described in Materials and Methods. Signal from cells treated with representative positive well 5D7 shown in black bar. (B) Cytotoxicity evaluation of well 5D7 supernatant and other internalization-positive cultures in serial dilution (left to right) following secondary expansion of masterwells. (C) Cytotoxicity evaluation of purified antibody from subsequently isolated clone 5D7 in combination with 2°ADC. Also shown are the 2°ADC alone, and 2°ADC in combination with internalizing mAbs OKT9 and BR96 and nonbinding control mAb AC10.

2°ADC system for discovery. Together with the results presented here this 2°ADC system is a highly useful screening tool to identify optimal antitumor mAbs for drug delivery.

LITERATURE CITED

(1) Naito, K., Takeshita, A., Shigeno, K., Nakamura, S., Fujisawa, S., Shinjo, K., Yoshida, H., Ohnishi, K., Mori, M., Terakawa, S., Ohno, R. (2000) Calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (gemtuzumab zogamicin, CMA-676) shows cytocidal effect on CD33-positive leukemia cell lines, but is inactive on P-glycoprotein-expressing sublines. *Leukemia 14*, 1436−43.

(2) Doronina, S. O., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cerveny, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., Senter, P. D. (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol. 21*, 778−784.

(3) Raub, T. J., and Roberts, R. M. (1986) Cell surface glycoproteins of CHO cells. II. Surface distribution and pathway of internalization. *Exp. Cell Res. 165*, 92−106.

(4) Titus, J. A., Haugland, R., Sharrow, S. O., and Segal, D. M. (1982) Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J. Immunol. Methods 50*, 193−204.

(5) Kondo, T., FitzGerald, D. J. P., Adhya, S., and Pastan, I. (1988) Activity of immunotoxins constructed with modified Pseudomonas exotoxin A lacking the cell recognition domain. *J. Biol. Chem. 263*, 9470−9475.

(6) Till M., May R. D., Uhr, J. W., Thorpe, P. E., and Vitetta, E. S. (1988) An assay that predicts the ability of monoclonal antibodies to form potent ricin A chain-containing immunotoxins. *Cancer Res. 148*, 1119−23.

(7) Bross, P. F., Beitz, J., Chen, G., Chen, X. H., Duffy, E., Kieffer, L., Roy, S., Sridhara, R., Rahman, A., Williams, G., and Pazdur, R. (2001) Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin. Cancer Res. 7*, 1490−6.

(8) Dubowchik, G. M. and Firestone, R. A. (1998) Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorg. Med. Chem Lett. 8*, 3341−6.

(9) Dubowchik, G. M., Radia, S., Mastalerz, H., Walker, M. A., Firestone, R. A., King, D. H., Hofstead, S. J., Willner, D., Lasch, S. J.; Trail PA. (2002) Doxorubicin immunoconjugates containing bivalent, lysosomally cleavable dipeptide linkages. *Bioorg. Med. Chem. Lett. 12*, 1529−32.

(10) Dubowchik, G. M., Mosure, K., Knipe, J. O. and Firestone, R. A. Cathepsin B-sensitive dipeptide prodrugs. 2. Models of anticancer drugs paclitaxel (Taxol), mitomycin C and doxorubicin. (1998) *Bioorg. Med. Chem. Lett. 8*, 3347−3352.

(11) Walker, M. A., Dubowchik, G. M., Hofstead, S. J., Trail, P. A. and Firestone, R. A. (2002) Synthesis of an immunoconjugate of camptothecin. *Bioorg. Med. Chem. Lett. 12*, 217−9.

(12) Francisco, J. A., Cerveny, C. G., Meyer, D. L., Mixan, B. J., Klussman, K., Chace, D. F., Rejniak, S. X., Gordon, K. A., DeBlanc, R., Toki, B. E., Law, C. L., Doronina, S. O., Siegall, C. B., Senter, P. D., and Wahl, A. F. (2003) AC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood 102*, 458−1465.

(13) Mariani, E., Mariani, A. R., Monaco, M. C., Lalli, E., Vitale, M., and Facchini, A. (1991) Commercial serum-free media: hybridoma growth and monoclonal antibody production. *J. Immunol. Methods. 145*, 175−183.

(14) Yelton, D. E., Desaymard, C., and Scharff, M. D. (1981) Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma 1*, 5−11.

(15) Aisenberg, A. C., and Wilkes, B. M. (1980) Unusual human lymphoma phenotype defined by monoclonal antibody. *J. Exp. Med. 152*, 1126−1131.

(16) Hellström, I., Garrigues, H., Garrigues, U., and Hellström, K. E. (1990) Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigen. *Cancer Res. 50*, 2183-2190.

(17) Yarnold, S., and Fell, H. P. (1994) Chimerization of antitumor antibodies via homologous recombination conversion vectors. *Cancer Res. 54*, 506−512.

(18) Bowen, M. A., Olsen, K. J., Cheng, L., Avila, D., and Podack, E. R. (1993) Functional effects of CD30 on a large granular lymphoma cell line, YT. Inhibition of cytotoxicity, regulation of CD28 and IL-2R, and induction of homotypic aggregation. *J. Immunol. 151*, 5896−906.

(19) Wahl, A. F., Klussman, K., Thompson, J. D., Chen, J. H., Francisco, L. V., Risdon, G., Chace, D. F., Siegall, C. B., and Francisco, J. A. (2002) The Anti-CD30 Monoclonal Antibody SGN-30 Promotes Growth Arrest and DNA Fragmentation in Vitro and Affects Antitumor Activity in Models of Hodgkin's Disease. *Cancer Res. 62*, 3736−42.

(20) Fell, H. P., Margit, A. G., Yelton, D., Lipsich, L., Schieven, G. L., Marken, J. S., Aruffo, A., Hellström, K. E., Hellström, I., and Bajorath, J. (1992) Chimeric L6 antitumor antibody. *J. Biol. Chem. 267*, 15552−15558.

(21) Pettit, G. R., (1997) The dolastatins. *Fortschr. Chem. Org. Naturst. 7*, 1−79.

(22) Nakayama, G. R., Caton, M. C., Nova, M. P., and Parandoosh, Z. (1997) Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *J. Immunol. Methodol. 204*, 205−208.

(23) Kohls, M. D., and Lappi, D. A. (2000) Mab-Zap: A tool for evaluating antibody efficacy for use in an immunotoxin. *Biotechniques 28*, 162−165.

(24) Hansen, S. H., Sandvig, K., and van Deurs, B. (1992) Internalization efficiency of the transferrin receptor. *Exp. Cell Res. 199*, 19−28.

(25) Hellström, I., Horn, D., Linsley, P., Brown, J. P., Brankovan, V., and Hellström, K. E. (1986) Monoclonal antibodies raised against human lung carcinoma. *Cancer Res. 46*, 3917−3923.

(26) Hellström, I., Beaumier, P. L., and Hellström, K. E. (1986) Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas. *Proc. Natl. Acad. Sci. U.S.A. 83*, 7059−63.

(27) Kerr, D. E., Wu, G. Y., Wu, C. H. and Senter, P. D. (1997) Listeriolysin O potentiates immunotoxin and bleomycin cytotoxicity. *Bioconjugate Chem. 8*, 781−784.

(28) Hellström, I., Brown, J. P., and Hellström, K. E. (1983) Melanoma-associated antigen p97 continues to be expressed after prolonged exposure of cells to specific antibody. *Int. J. Cancer 31*, 553−555.

(29) Siemers, N. O., Kerr, D. E., Yarnold, S., Stebbins, M. R., Vrudhula, V. M., Hellström, I., Hellström, K. E., and Senter, P. D. (1997) Construction, expression, and activities of L49-sFv-beta-lactamase, a single-chain antibody fusion protein for anticancer prodrug activation. *Bioconjugate Chem. 8*, 510−519.

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