Lysosomal Trafficking and Cysteine Protease Metabolism Confer Target-specific Cytotoxicity by Peptide-linked Anti-CD30-Auristatin Conjugates*

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The chimeric anti-CD30 monoclonal antibody cAC10, linked to the antimitotic agents monomethyl auristatin E (MMAE) or F (MMAF), produces potent and highly CD30-selective anti-tumor activity in vitro and in vivo. These drugs are appended via a valine-citrulline (vc) dipeptide linkage designed for high stability in serum and conditional cleavage and putative release of fully active drugs by lysosomal cathepsins. To characterize the biochemical processes leading to effective drug delivery, we examined the intracellular trafficking, internalization, and metabolism of the parent antibody and two antibody–drug conjugates, cAC10vc-MMAE and cAC10vc-MMAF, following CD30 surface antigen interaction with target cells. Both cAC10 and its conjugates bound to target cells and internalized in a similar manner. Subcellular fractionation and immunofluorescence studies demonstrated that the antibody and antibody-drug conjugates entering target cells migrated to the lysosomes. Trafficking of both species was blocked by inhibitors of clathrin-mediated endocytosis, suggesting that drug conjugation does not alter the fate of antibody-antigen complexes. Incubation of cAC10vc-MMAE or cAC10vc-MMAF with purified cathepsin B or with enriched lysosomal fractions prepared by subcellular fractionation resulted in the release of active, free drug. Cysteine protease inhibitors, but not aspartic or serine protease inhibitors, blocked antibody-drug conjugate metabolism and the ensuing cytotoxicity of target cells and yielded enhanced intracellular levels of the intact conjugates. These findings suggest that in addition to trafficking to the lysosomes, cathepsin B and perhaps other lysosomal cysteine proteases are requisite for drug release and provide a mechanistic basis for developing antibody-drug conjugates cleavable by intracellular proteases for the targeted delivery of anti-cancer therapeutics.

Cancer is the second leading cause of mortality in the United States, resulting in >500,000 American deaths annually. The unmet medical need for more effective anticancer therapeutics, especially for strategies that can focus toxicity to tumor cells and away from normal tissues, has lead to the development of monoclonal antibodies (mAbs) linked to immunotoxins, radionuclides, or cytotoxic drugs, to provide selective elimination of antigen-positive target cells. The first such clinically approved agent, Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 mAb linked to the potent DNA damaging agent calicheamicin, is used for the treatment of patients with relapsed acute myeloid leukemia (1). Mylotarg and multiple other mAb–drug conjugates (ADCs) and mAb-toxin conjugates currently in development use primarily disulfide or hydrazone linkers sensitive to the reductive or acidic environment of the tumor cell (2–6). These linkers readily deliver and liberate free drug or toxin within the target cell and yet are relatively unstable in circulation compared with the circulating half-life of the mAb (7), resulting in premature drug release.

An alternative approach is to employ ADC linkers of protease-cleavable dipeptides. These combine qualities of high stability in serum or plasma with efficient drug release potentially by lysosomal proteases (8). Using this strategy, we recently described a new ADC of the anti-CD30 mAb cAC10 (9), appended to the anti-tubulin agent, monomethyl auristatin E (MMAE), via a cathepsin-cleavable valine-citrulline (vc) linker (10, 11). This drug linker system was shown to be highly stable in vitro and in vivo (7, 12), and when applied to multiple mAbs, the resulting ADCs were selectively potent and effective against cognate antigen-positive tumor cells and tumor xenografts (LeY (8), CD30 (11), TMEFF2 (13), CD20 (14), and EphB2 (15)). One premise of this drug delivery technology is that the mAb-antigen complex on the cell surface will internalize, traffic to the lysosomes, and be metabolized by lysosomal proteases to release free drug.

ADC efficacy therefore depends in part on mAb-antigen interaction at the cell surface triggering the internalization, trafficking, and subsequent release of the active cytotoxic payload. Thus, conjugates comprised of different drug linkers or with different mAbs against the same target can vary significantly in their utility. For example, anti-CD20 ADC incorporating doxorubicin (16, 17) or ricin-A (18) were ineffective as anti-tumor agents, whereas anti-CD20 conjugates of vcMMAE were highly effective against CD20− tumors (14). Interestingly, the anti-CD20 mAb remained on the cell surface, whereas the anti-CD20vcMMAE conjugate was readily internalized, resulting in rapid cell cycle arrest and apoptosis (14). Alternatively, in targeting CD30, both mAb and anti-CD30 ADC demonstrated comparable binding and internalization rates in CD30+ tumor cells (11), with the ADC inducing rapid mitotic arrest cell and apoptosis (11). Just what governs optimal ADC internalization and trafficking critical for effective drug release is not known.

Here we examine the trafficking and intracellular fate of anti-CD30 mAb cAC10 and two cAC10-drug conjugates, cAC10vc-MMAE and cAC10vc-MMAF, in CD30+ T-cell lymphoma cell lines. Using flow cytometry, immunofluorescence, subcellular fractionation, and chemical inhibitors of trafficking and processing, we demonstrate that the
cytotoxicity of peptide-linked auristatin ADCs is contingent upon their delivery to the lysosome and the activity of lysosomal cysteine proteases. These studies demonstrate the functional basis of drug delivery by ADCs with protease-cleavable linkers for anti-cancer therapeutics.

**MATERIALS AND METHODS**

**Flow Cytometry for Antibody and Antibody-Drug Conjugate Internalization and Trafficking**—The chimeric anti-CD30 antibody cAC10 was produced as described previously (9) and conjugated to MMAE (11) and monomethyl auristatin F (MMAF) (19) to yield cAC10vc-MMAE and cAC10vc-MMAF, respectively. The CD30+ Hodgkin disease L540cy cell line, a derivative of the L540 Hodgkin disease cell line adapted for xenograft growth, was provided by Dr. Harald Stein (Institut für Pathologie, University of Veinikum Benjamin Franklin, Berlin, Germany). L540cy cells were grown in RPMI 1640 supplemented with 20% heat-inactivated fetal calf sera and antibiotics.

L540cy cells (1 × 10⁶ cells/ml) were incubated with 2 μg/ml cAC10, cAC10vc-MMAE, or cAC10vc-MMAF for 30 min on ice, rinsed with ice-cold PBS, and then incubated for 30 min in the presence (cross-linking) or absence (no cross-linking) of 2 μg/ml goat anti-human IgG (Jackson Immunoresearch, West Grove, PA) (20). The cells were rinsed with cold PBS, resuspended in growth media, and incubated at 37 °C. The samples were harvested at various times and processed for flow cytometry. To detect surface-bound ADC, the cells were incubated with 10 μg/ml mouse anti-id antibody to cAC10 for 30 min at 4 °C, washed, and then incubated with 10 μg/ml goat anti-mouse IgG-fluorescein isothiocyanate with minimal cross-reactivity to human IgG (Fcγ-specific, F(ab)2 fragment; Jackson Immunoresearch). To detect internalized antibody or ADC, the cells were washed with cold PBS, incubated with proteinase K (5 μg/ml for 10 min at 37 °C), washed to remove cell surface-bound antibody, and treated with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) prior to incubation with the anti-id antibody as described above. The cells were assessed by flow cytometry using a Becton Dickinson FACScan.

In other experiments, L540cy cells were preincubated with subcellular trafficking inhibitors (21–27) (10 μM colchicine, 0.5 μM amantadine, 0.1 μM phenylarsine oxide, 20 μM clasto-lactacystin-β-lactone, 40 μM cytochalasin D, 3 mM ammonium chloride, 14 μg/ml chlorpromazine, 10 μg/ml nystatin; Sigma) or cysteine protease inhibitors (3 μM CA074-OME and 20 μM E64d; Calbiochem, San Diego, CA) for 30 min at 4 °C prior to a 3- or 5-h incubation with cAC10 or cAC10-drug conjugates at 37 °C. The cells were processed as described above for internalized antibody except that detection was done by staining with a goat anti-human IgG-fluorescein isothiocyanate-labeled secondary antibody (Jackson Immunoresearch). Quantitative evaluation of the surface expression of CD30 on the Hodgkin disease cell line L540cy and the anaplastic large cell lymphoma cell line (ALCL) Karpas-299 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GMBH) was performed using the QiFiKit bead assay (DAKO, Carpinteria, CA).

**Immunofluorescence for Antibody and Antibody-Drug Conjugate Trafficking**—L540cy cells (5 × 10⁵ cells/ml in regular medium) were incubated with 1 μg/ml cAC10, cAC10vc-MMAE, or cAC10vc-MMAF for 30 min on ice or for 16 h at 37 °C. After the incubation, the cells were washed with cold PBS to remove unbound antibody and drug conjugates. The cells were fixed and permeabilized with Cytofix/Cytoperm and stained as described previously (14). cAC10 and its conjugates were detected following incubation with Alexa Fluor 488-labeled goat anti-human IgG (H+L) with minimal cross-reactivity to mouse IgG (Molecular Probes, Eugene, OR). Lysosomal compartments were visualized by staining with Lamp-1 (mouse CD107a antibody, BD Biosciences) and a secondary antibody, Alexa Fluor 568-conjugated goat anti-mouse IgG (H+L) with minimal cross-reactivity to human IgG (Molecular Probes). Nuclear compartments were stained with 4',6'-diamidino-2-phenylindole (Roche Applied Science). Fluorescence images were acquired with a Leitz Orthoplan epifluorescence microscope. In other experiments, the cAC10 antibody was linked to Alexa Fluor 488 reactive dye (Molecular Probes) and incubated with L540cy cells (2 × 10⁶ cells/ml) at 200 ng/ml for 3 h at 37 °C in the presence or absence of trafficking inhibitors (40 μM cytochalasin D or 3 mM ammonium chloride). Lysosomal and nuclear compartments were visualized by staining with LysoTracker and Hoescht DNA dyes, respectively (Molecular Probes). Fluorescence images were taken on fixed cells with a Carl Zeiss Axiovert 200M microscope.

**Western Blot Analyses**—To evaluate the metabolism of cAC10-drug conjugates, purified cathepsin B (2 units/ml; Calbiochem) was incubated for 3 h at 37 °C with 5 μg/ml cAC10vc-MMAE or cAC10vc-MMAF in buffer (2 mM dithiothreitol, 50 mM sodium acetate, pH 5.0) in the presence or absence of 10 μM E64d cysteine protease inhibitor. In other experiments, Karpas and L540cy pooled lysosome-enriched fractions (numbers 10–19) were incubated with 5 μg/ml cAC10vc-MMAE for 18 h in the presence or absence of 20 μM E64d or 3 μM CA074-Ome. The reactions were stopped by quick freezing in a dry ice bath. The digests were mixed with Novex sample buffer (Invitrogen), run on 4–20% Tris-Gly or 4–12% Bis-Tris gradient gels (Invitrogen) under reducing conditions, and transferred onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked with 2% nonfat dry milk in PBST (PBS+ 0.1% Tween 20) prior to incubation with the mouse SG2.15 antibody to detect drug. This antibody recognizes both MMAE and MMAF (data not shown). Detection was then performed using horseradish peroxidase–goat anti-mouse IgG (Fcγ-specific, Jackson Immunoresearch) followed by ECL (SuperSignal West Pico; Pierce). To detect the heavy and light chains of the antibody, detection was performed using horseradish peroxidase–goat anti-human IgG (Fcγ specific; Jackson Immunoresearch) or horseradish peroxidase–goat anti-human IgG (κ-specific; Southern Biotech, Birmingham, AL), respectively, followed by ECL.

**Cytotoxicity Assay**—Cell viability was measured by Alamar Blue (BIOSOURCE International, Camarillo, CA) dye reduction (10, 14) or with CellTiter-Glo (Promega, Madison, WI). The results were reported.
as the IC_{50} values, the concentration of compound needed to yield a 50% reduction in viability compared with untreated cells (control /H11005 100%).

To inhibit ADC metabolism, L540cy cells (8 /H11003 10^3 cells/well) were pretreated with 20 /H9262 M E64d, 0.5 /H9262 M CA074-OME, 10 /H9262 M calpeptin (Calbiochem), 3 /H9262 M N-acetyl-Leu-Leu-Nle-CHO (calpain inhibitor I; Calbiochem), 20 /H9262 g/ml pepstatin A (Calbiochem), 20 /H9262 M p-aminoethylbenzenesulfonyl fluoride (Calbiochem), 20 /H9262 g/ml aprotinin (Sigma), or 25 /H9262 M cystamine dihydrochloride (Sigma) for 1 h prior to the addition of cAC10vc-MMAE. The cultures were maintained for 96 h prior to the addition of Celltiter-Glo. The cells were incubated with Celltiter-Glo reagent for 25 min, and the dishes were processed for luminescent readout.

In other experiments, Karpas cells (5 × 10^5 cells) were incubated in growth medium for 4 h at 37 °C prior to the harvesting of cells to generate lysosomal fractions. The fractions were washed with 0.25 M sucrose buffer containing 10 mM HEPES, pH 7.2. Each fraction was incubated with cAC10vc-MMAE (5 μg/ml) for 24 h in reaction buffer (2 mM dithiothreitol, 50 mM sodium acetate, pH 5.0). Afterward, the fractions were diluted 300-fold in RPMI growth medium and added to cultures of CD30-negative Ramos cells (1 × 10^6 cells/well; American Type Culture Collection, Manassas, VA) in the presence or absence of 2.5 μg/ml mouse anti-MMAE antibody, SG3.190. Cell growth was assessed 96 h later with Alamar Blue. The cells were incubated with the dye for 4 h prior to fluorescence measurement on a Fusion HT plate reader.

**RESULTS**

MMAE and MMAF belong to the dolastatin 10 family of highly potent anti-mitotic agents that inhibit tubulin polymerization (Fig. 1) (10, 19). In contrast to MMAE, the charged, carboxylic acid terminus of free MMAF can potentially limit passive transit through cell membranes. The cAC10 antibody-drug conjugates of MMAE and MMAF containing a protease-cleavable vc linker were prepared as described previously (10, 19).

**TABLE 1**

| Cell line  | Type            | CD30* | MMAE  | MMAF  | cAC10vc-E | cAC10vc-F |
|------------|-----------------|-------|-------|-------|-----------|-----------|
| Karpas ALCL (T-cell) | 290,676         | 0.52 ± 0.29^a  | 101 ± 12 | 0.11 ± 0.016^d  | 0.05 ± 0.005 |
| L540cy HD (T-cell)  | 587,511         | 1.25 ± 0.81^b  | 65.6 ± 14.3 | 0.32 ± 0.032^d  | 0.11 ± 0.035 |
| Ramos Burkitt lymphoma | 0               | 0.039 ± 0.02^b  | 58 ± 10  | >75      | >75       |

* CD30 levels expressed as the number of antibody molecules bound per cell.

^a p < 0.005 to MMAE.

^b p < 0.001 to MMAE.

^c p < 0.001 to cAC10vc-F.

Cysteine Protease Activation of Anti-CD30-Drug Conjugates

FIGURE 1. Structures of vc-MMAE and vc-MMAF used for mAb conjugation. The structures of each drug appended to mAb sulphydryl groups via valine-citrulline linkers are depicted. ADCs were prepared by controlled partial reduction of internal mAb disulfides with dithiothreitol, followed by the addition of the linker drugs as described previously (10, 19).

| Cytotoxic activities of cAC10vc-MMAE and cAC10vc-MMAF on CD30^+ tumor cell lines |
|-------------------------------|----------------|---------|---------|---------|---------|---------|
| Cell line  | Type            | CD30^+ | MMAE  | MMAF  | cAC10vc-E | cAC10vc-F |
|------------|-----------------|-------|-------|-------|-----------|-----------|
| Karpas ALCL (T-cell) | 290,676         | 0.52 ± 0.29^a  | 101 ± 12 | 0.11 ± 0.016^d  | 0.05 ± 0.005 |
| L540cy HD (T-cell)  | 587,511         | 1.25 ± 0.81^b  | 65.6 ± 14.3 | 0.32 ± 0.032^d  | 0.11 ± 0.035 |
| Ramos Burkitt lymphoma | 0               | 0.039 ± 0.02^b  | 58 ± 10  | >75      | >75       |
Cysteine Protease Activation of Anti-CD30-Drug Conjugates

L540cy cells were preincubated with mAb or ADCs and harvested at various incubation times for flow cytometry as described under "Materials and Methods." A time-dependent increase in intracellular levels of mAb and ADC in L540cy cells. Cell surface-bound mAb or ADC (Total, filled symbols) decreased with time, whereas intracellular levels (open symbols, dotted lines) rose to a plateau at ~5 h. The data shown (means ± S.D.) are expressed as percentages of the total mean fluorescence intensities (MFI) corrected for background (cells only, IgG isotype) from three independent experiments. Total mean fluorescence intensities was determined from cells held at 4 °C. B, cross-linking enhanced intracellular levels of mAb and ADC. The levels of internalized mAb or the ADCs were greater in cells incubated with anti-human IgG (cross-linking, closed symbols) compared with those exposed to mAb or ADC only (open symbols). Peak intracellular levels of mAb and ADCs were achieved between 2 and 5 h. The data are shown (means ± S.D.) as the percentage mean fluorescence intensities, corrected for background, from three to five independent experiments. E, MMAE; F, MMAF; X-link, cross-linked.

FIGURE 2. Internalization and cellular trafficking of cAC10 and cAC10-drug conjugates. L540cy cells were preincubated with mAb or ADCs and harvested at various incubation times for flow cytometry as described under "Materials and Methods." A, time-dependent increase in intracellular levels of mAb and ADC in L540cy cells. Cell surface-bound mAb or ADC (Total, filled symbols) decreased with time, whereas intracellular levels (open symbols, dotted lines) rose to a plateau at ~5 h. The data shown (means ± S.D.) are expressed as percentages of the total mean fluorescence intensities (MFI) corrected for background (cells only, IgG isotype) from three independent experiments. Total mean fluorescence intensities was determined from cells held at 4 °C. B, cross-linking enhanced intracellular levels of mAb and ADC. The levels of internalized mAb or the ADCs were greater in cells incubated with anti-human IgG (cross-linking, closed symbols) compared with those exposed to mAb or ADC only (open symbols). Peak intracellular levels of mAb and ADCs were achieved between 2 and 5 h. The data are shown (means ± S.D.) as the percentage mean fluorescence intensities, corrected for background, from three to five independent experiments. E, MMAE; F, MMAF; X-link, cross-linked.

FIGURE 3. Trafficking of cAC10 and cAC10-drug conjugates to the lysosomes in L540cy cells. The cells were incubated with the mAb or ADCs on ice or at 37 °C. Cell surface and intracellular mAb or ADC were visualized by fluorescence microscopy. cAC10 antibody or ADC is shown in green; the lysosomal markers Lamp-1 or LysoTracker are shown in red; and 4′,6′-diamidino-2-phenylindole or Hoechst-stained nuclei is in blue. Co-localization of signals for antibody or ADC with Lamp-1 (16 h, arrows) or LysoTracker (3 h, Merged) is shown by the yellow signal. The results for the ADCs at 3 h were similar to those shown for cAC10. The data shown are representative of results from two independent experiments. Magnification bar, 10 microns.

comparison of free drugs showed that the cell-permeable MMAE was 50–200-fold more effective than MMAF. As an ADC however, cAC10vc-MMAF was significantly more potent than cAC10vc-MMAE (IC50 < 0.11 nM, p < 0.001). Neither drug conjugate exhibited appreciable activity against multiple antigen-negative cell lines, demonstrating that the potency of these ADCs is antigen-dependent. For example, the IC50 value against the CD30-negative cell line Ramos following 96 h continuous exposure was >75 nM (Table 1).

To evaluate the trafficking and internalization of cAC10 and its ADC, CD30+ L540cy cells were preincubated with mAb or ADCs and treated with vehicle or anti-human IgG for cross-linking prior to culture at 37 °C. Cross-linked antibodies exhibit increased internalization and cellular clearance (10). At appointed times, the cells were harvested and assessed for surface-bound and intracellular levels of mAb and ADCs by flow cytometry (Fig. 2). Cell surface levels of both mAb and ADCs decreased sharply with time, coincident with increased intracellular levels (Fig. 2), suggesting that they internalized with similar kinetics. By 20 h, the surface levels of mAb and ADCs were ~60% of the initial levels (Fig. 2A). Intracellular levels rose quickly within the first hour, peaked between 2 and 5 h, and maintained lower but steady levels up to 48 h (Fig. 2B). Cross-linking of the cAC10 antibody and ADCs increased intracellular levels of both the mAb and the conjugates by ~3-fold over levels observed in the absence of cross-linking (Fig. 2B).

Immunofluorescence microscopy was used to localize the internalized mAb and ADCs within L540cy cells (Fig. 3). The cells were incubated with the mAb or ADCs either on ice or at 37 °C for the stated times and imaged immediately or fixed, permeabilized, and then processed for immunofluorescence. The cells incubated on ice and stained for mAb or ADCs (Fig. 3, top panels, green signal) showed diffuse, cell surface-associated staining and no evidence of internalization. Lysosomes, visualized using an antibody to lysosome-associated membrane protein 1 (Lamp-1; Fig. 3, top panels, red signal) or with LysoTracker (bottom panels, red signal) were distinct and punctate. At 37 °C, there was capping and punctate staining for both mAb and ADCs within the L540cy cells and reduced staining on the cell surface. The intracellular mAb and ADC signals co-localized with those for Lamp-1 (Fig. 3, arrows, yellow signal, 16 h incubation) or with LysoTracker (Fig. 3, 3-h incubation, yellow signal, Merged, and data not shown), suggesting that
both mAb and ADCs internalized and were transported to the lysosomes. The results were not affected by fixation and sample processing because similar images were obtained using either live or fixed cells.

 Trafficking of the ADCs to the lysosomes was also confirmed biochemically. L540cy cells were preincubated with Alexa Fluor-labeled cAC10vc-MMAE. The cells were then lysed and organelles fractionated by density gradient as described under "Materials and Methods." Enrichment for lysosomes following ultracentrifugation was confirmed.

**FIGURE 4. Lysosomal localization of ADCs and metabolism by cysteine proteases.**

**A.** Internalized ADC co-migrates with lysosomal β-galactosidase activity. CD30⁺ L540cy cells were preincubated with Alexa Fluor 488-labeled cAC10vc-MMAE, lysed, and fractionated on Optiprep density gradients. The resultant cell fractions were assayed for β-galactosidase activity and for the labeled ADC (RFU, relative fluorescence units). The data shown are representative of results from two separate experiments. B, cysteine protease and trafficking inhibitors block ADC metabolism. L540cy cells were preincubated with inhibitors 30 min prior to the addition of the mAb or ADCs. Intracellular levels of mAb or ADC were determined by flow cytometry at 5 h after exposure. Intracellular levels of mAb or ADCs were significantly diminished in cells treated with inhibitors of clathrin-mediated endocytosis or lysosomal activity (NH₄Cl) but enhanced in cells treated with inhibitors of cysteine proteases. Similar results were obtained with a 3-h incubation. The data (means ± S.D.) are expressed as the percentage of untreated control values from three separate experiments. *, p < 0.001 to untreated cells for either mAb or ADC. ND, not determined; PAO, phenylarsine oxide; Clasto, clastolactacystin-β-lactone; Chlorm, chlorpromazine; Cyto D, cytochalasin D. C, trafficking of cAC10 mAb in L540cy cells is affected by NH₄Cl and cytochalasin D. L540cy cells were treated with vehicle (control), NH₄Cl, or cytochalasin D 30 min prior to a 3-h incubation with Alexa Fluor 488-labeled cAC10. Cell surface and intracellular mAb was visualized by fluorescence microscopy. cAC10 antibody is shown in green; LysoTracker lysosomal marker is in red; and Hoescht-stained nuclei are in blue. Co-localization of signals for mAb with LysoTracker is shown in yellow. The data are representative of results from two independent experiments. Magnification bar, 10 microns. D, Western analyses of cathepsin B digests of cAC10vc-MMAE and cAC10vc-MMAF. ADCs were incubated with purified cathepsin B for 3 h at 37 °C, and the samples were analyzed by Western blot with anti-drug antibody (SG2.15), goat anti-human IgG (Fcγ-specific), or goat anti-human IgG (κ-specific). Cathepsin B removed drug on the antibody frame. Loss of drug was blocked by the protease inhibitor, E64d. E, cAC10vc-MMAE; F, cAC10vc-MMAF; HC, heavy chain; LC, light chain.
by determining the distribution of phenotypic markers for various subcellular organelles. The markers for nuclei (DNA) and mitochondria (ATP generation) were found in the more dense fractions of the gradient (fractions 1–7), whereas the lysosomal marker, β-galactosidase activity, was detected in the middle fractions (fractions 8–16), and the late endosomes/Golgi may be associated with lighter fractions (fractions 16–20), as reported elsewhere (29).

When gradient fractions were analyzed for the levels of fluorescently labeled ADC by fluorometry and for β-galactosidase activity (Fig. 4A, peak enzyme activity was coincident with that of the fluorescently labeled ADC. Consistent with data from photomicrography (Fig. 3), these findings suggest that the internalized ADCs traffic and concentrate in the lysosomes.

To assess the prevailing mechanism of cellular uptake, the cells were preincubated with inhibitors of trafficking prior to the addition of the mAb or ADCs and subsequent evaluation by flow cytometry (Fig. 4B) and fluorescence microscopy (Fig. 4C). Ammonium chloride (NH₄Cl), a lysosomotropic agent, disrupts trafficking and lysosomal processing by neutralizing the acidic environment of the endosomal/lysosomal compartments (30–32). Treatment of L540cy cells with this reagent significantly decreased the total intracellular levels of the mAb and ADCs (Fig. 4B, p < 0.001; Fig. 4C, yellow signal) and increased the cell surface levels (Fig. 4C, green signal). Intracellular accumulation of mAb and ADCs was also significantly diminished (40–65%) in cells pretreated with inhibitors of clathrin-mediated endocytosis (amantadine, phenylarsine oxide, clasto-lactacystin-B, and chlorpromazine) (21, 25, 27) (Fig. 4B, p < 0.001), including cytochalasin D (23, 27), that disrupt actin assembly. Increased levels of the antibody and ADCs were found on the cell surface of cytochalasin D-treated cells (Fig. 4C). Conversely, inhibitors of cathepsin B-mediated proteolysis (CA074-OME and E64d) (24) significantly enhanced the mAb and ADC levels by 50–65% (Fig. 4B, p < 0.001). Under the conditions used, compounds known to modulate caveolae-mediated uptake (e.g. nystatin or filipin) (26) or microtubule assembly (e.g. colchicine or nocodazole) (22) had no effect on the total intracellular levels of the mAb and ADCs (Fig. 4B and data not shown). These data collectively suggest that the mAb and ADCs internalize and traffic to the lysosomes with similar magnitude and kinetics via clathrin-mediated endocytosis. Additionally, the activity of lysosomal enzymes such as cathepsin B appeared to modulate the intracellular levels of ADC.

We then investigated the ability of lysosomal metabolism of ADCs to release free drug. In a control study, ADCs were incubated with the purified lysosomal cysteine protease, cathepsin B (33). The reaction products were analyzed by Western blot using antibodies to the drug and to the heavy and light chain components of the ADC. Fig. 4D shows that cathepsin B treatment effectively removed detectable drug associated with the heavy and light chains. Cleavage of drug from the heavy and light chains of cAC10vc-MMAE and cAC10vc-MMAF with purified cathepsin B led to slight mobility shifts (reduced mass) but nonetheless preserved discrete heavy and light chain bands (Fig. 4D). No heterogeneity caused by mAb proteolysis was detected. These findings agree with those reported previously (10). Interestingly, an increase in signal for the light chain was observed in the presence of cathepsin B. This may be due to limited proteolysis augmenting the immunoreactivity of the κ-specific detection antibody (Fig. 4D). In addition, cysteine protease inhibitors such as E64d and CA074-OME were able to block the metabolism of the antibody and ADCs, restoring the drug-associated signal on Western analysis (Fig. 4D) as well as increasing the intracellular levels of these proteins (Fig. 4B).

To determine whether diminished ADC metabolism was correlated with reduced cytotoxicity, we evaluated the effects of cAC10vc-MMAE on the viability of L540cy cells in the presence of the cysteine protease inhibitors, E64d and CA074-OME. In L540cy cells, CA074-OME alone had small effects on the cytotoxic activity of cAC10vc-MMAE (Fig. 5A, diamonds), shifting the IC₅₀ ~1.7-fold. In comparison, E64d significantly reduced the activity of the ADC by shifting the IC₅₀ ~10-fold (Fig. 5A, triangles). A greater decrease in the activity of the ADC was observed when the cells were pretreated with both inhibitors. With the combination of E64d and CA074-OME, there was a marked increase in the number of viable cells and a dramatic shift in IC₅₀ to greater than 10 µg/ml ADC or 250 nM drug (Fig. 5A, open circles). When E64d was added 6 or 18 h after the start of culture with the ADC, there was minimal blocking effect on the activity of the ADC (data not shown). This was not surprising given that anti-CD30-ADCs appear to work rapidly. The cAC10vc-MMAE drug conjugate induced the growth arrest of L540cy cells within 12 h (11). At sufficient levels (>60 µM for E64d and >3 µM for CA074-OME), the protease inhibitors were cytotoxic. Yet treatment of L540cy cells with subtoxic levels of these inhibitors reduced the activity of the ADC, resulting in an increase in the IC₅₀ values and an increase in the viability of the lymphoma cells. In comparison, inhibitors of serine proteases (aprotinin and p-a2-macroglobulin zenzesulfonyl fluoride), aspartic proteases (pepstatin A), transglutaminases (cystamine (34)) or cytoskeleton-associated calpains (calpeptin and N-acetyl-Leu-Leu-Nle-CHO (35)) had no significant effects on the activity of cAC10vc-MMAE (Fig. 5B) or cAC10vc-MMAF (data not shown).

We then explored the ability of subcellular fractions to process ADCs using lysosome-enriched fractions from another CD30⁺ cell line, Karpas 299 cells. The fractions were incubated with cAC10vc-MMAE and then analyzed by Western blot using an anti-MMAE antibody to detect drug associated with the heavy and light chains of the cAC10 antibody. Fig. 5C clearly shows a reduced signal corresponding to the loss of drug associated with both mAb heavy and light chains that was coincident with fractions containing the peak lysosomal β-galactosidase activity (fractions 13–17). Drug loss from the mAb chains was blocked in the presence of E64d or CA074-OME (Fig. 5D for cAC10vc-MMAE and data not shown for cAC10vc-MMAF). To determine whether the released drug was biologically active, aliquots of these reaction mixtures were added to the CD30-negative Ramos cell line with cytotoxicity assessed after 96 h. The released drug is freely permeable and cytotoxic to nontarget cells, whereas intact ADC is not. Fig. 5E shows a significant reduction in the viability of Ramos cells coincident with the peak of lysosomal enzyme activity in fractionated Karpas cells (fractions 13–17). Cytotoxicity was blocked by the presence of an anti-drug antibody, confirming that the effect was due to the presence of active drug in these fractions. Other fractions yielded reduced or negligible effects. The results collectively show that the cAC10-drug conjugates traffic to the lysosomes in target cells and interact with lysosomal enzymes, yielding the release of active free drug.

**DISCUSSION**

Targeted therapy offers the promise of selective elimination of anti-gen-positive tumor cells with minimal toxicity to normal tissues. To be therapeutically beneficial, the targeting mAb must efficiently internalize, traffic, and be metabolized to release a fully active agent. The development of a mAb-drug linker that is highly stable to the extracellular environment (7, 10, 12) yet readily releases fully active drug upon internalization into tumor cells has shown preclinical efficacy with mAbs against multiple targets in several cancers (10, 11, 13, 15). Here we examined the activities and the cellular properties of drug conjugates of
the anti-CD30 mAb, cAC10, appended to the anti-mitotic drugs, MMAE or MMAF, via the protease-cleavable valine-citrulline linker. In vitro cytotoxicity assays have shown that both conjugates were highly potent against CD30+/H11001 Karpas and L540cy cells. In L540cy cells, both mAb and ADC internalized via clathrin-mediated endocytosis and localized to the lysosomes. Inhibitors of caveolin-associated pathways did not appear to affect the CD30 internalization process, although caveolin may play a role in the internalization of other mAb-antigen complexes (14, 36, 37). As measured by the decline in cell surface quantities and increased intracellular levels of mAb, the internalization of both anti-CD30 mAb and ADCs was gradual and comparable. In contrast, vcMMAE appended to the anti-CD20 mAb rituximab significantly increased internalization rates compared with mAb alone (14), suggesting that ADC internalization is both mAb- and antigen-dependent. Approximately 40% of the initial surface-bound anti-CD30 mAb and ADC were lost by 20 h, without a concomitant increase in internalized signal. mAb or ADC within the cell was maximal between 2 and 5 h and remained constant over 48 h, suggesting that surface CD30 shedding into the medium (38) may contribute to a loss in extracellular detection. The repopulation of the cell surface and intracellular degradation of mAb may also play a role in maintaining steady state levels.

FIGURE 5. Lysosomal protease activity is necessary for release of active drug. A, cysteine protease inhibitors block the activity of cAC10vc-MMAE in L540cy cells. L540cy cells were treated for 1 h with the cysteine protease inhibitors E64d and CA074-OME prior to the addition of the ADC. Cell viability was assessed 96 h later. The data are expressed as the means ± S.D. of triplicate values (percentage of untreated control) from four separate experiments. B, minimal effects of inhibitors of aspartic proteases, serine proteases, transglutaminase, and calpains on the activity of cAC10vc-MMAE in L540cy cells. L540cy cells were incubated with the ADC in the presence or absence of serine protease inhibitors (aprotinin and p-aminobenzylbenzenesulfonyl fluoride), aspartic protease inhibitor (pepstatin A), calpain inhibitors (calpeptin and N-acetyl-Leu-Leu-Nle-CHO), a transglutaminase inhibitor (cystamine), or E64d. Cell viability was assessed 96 h later. Similar results were obtained with cAC10vc-MMAF. The data are expressed as the means ± S.D. of triplicate values (percentage of untreated control) from three separate experiments. C, drug release from cAC10vc-MMAE is coincident with lysosomal activity. CD30-positive Karpas cells were incubated with cAC10vc-MMAE. Lysosome-enriched fractions were prepared by density gradient sedimentation. Fractions from the gradient were analyzed for β-galactosidase activity and loss of mAb-associated MMAE drug by Western blot analysis. The first lane in the Western blot represents untreated ADC. The data shown are representative of the results from two independent experiments. D, lysosomal cysteine protease activity is blocked by E64d and CA074-OME. Lysosome-enriched fractions from Karpas cells (fractions 10–19; C) were pooled and incubated with cAC10vc-MMAE in the presence or absence of E64d and CA074-OME. The loss of mAb-associated MMAE drug was assessed by Western blot analysis. Similar results were obtained with cAC10vc-MMAF and with lysosomal fractions from L540cy cells. E, lysosomal incubation of cAC10vc-MMAE resulted in the release of active free drug. Gradient fractions from Fig. 5C were added to CD30-negative Ramos cells with or without a neutralizing anti-drug antibody. The cells were incubated for 96 h, and viability was assessed with Alamar Blue. Fractions containing peak β-galactosidase activity yielded maximum cytotoxic activity (squares). Cytotoxic activity was blocked by the presence of anti-drug antibody (E+anti-MMAE mAb, triangles). The data shown are representative of triplicate measurements (percentage of untreated control) from two separate experiments. $E = cAC10vc$-MMAE.
linked conjugates are highly stable in vitro and in vivo (7, 10, 12). In CD30 \(^{-}\) LS40cy cells, the blocking of either the internalization process or the activity of lysosomal enzymes, but not the activity of cytosolic enzymes, effectively reduced the intracellular levels of the ADCs and ablation of tumor cells. Within the lysosomes, cysteine proteases including cathepsin B but not aspartic or serine proteases or transglutaminases contributed to the release of active drug. Because released drugs are permeable and active against all cells, the results of the cytotoxicity assay using the CD30-negative Ramos cells clearly correlated with the lysosomal activity of Karpas cells with release of an active agent.

Inhibition of this cytotoxicity by a MMAE/MMAF-specific neutralizing antibody suggested that active drug and not other metabolites are released by the lysosomal activity. Other ADCs have shown a disconnect between trafficking to the lysosomes and ineffectiveness as a cytotoxic agent, suggesting that internalization, production, and concentration of free drug were inadequate to induce toxicity. Both of the anti-CD20 conjugates of vc-doxorubicin and vc-MMAE internalized and localized to the lysosomes of target cells but only the MMAE conjugate actively induced growth arrest and apoptosis (14), indicating that free doxorubicin was not efficiently released or sequestered (14). The current data demonstrate that whereas antigen expression levels may be sufficient to produce effective cell killing by ADC, the combined parameters of internalization, trafficking, lysosomal protease activity, drug release, and intracellular concentration of free drugs are key to determining the activity of ADCs against target tumor cells.

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