Intracellular Targeting with Low pH-triggered Bispecific Antibodies*

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Bispecific antibodies were designed to deliver a reversibly bound ligand into target cells and then spontaneously release it upon passage into acidified vesicles. These reagents were assembled by coupling monoclonal antibodies that recognize acid-sensitive epitopes on diphtheria toxin to cell type-specific monoclonal antibodies. The dual binding capacity of the bispecific antibodies was confirmed by delivery of 125I-diphtheria toxin to target molecules present on intact cells. Bispecific antibodies directed against transferrin receptors on human cells were loaded with toxin and tested for cytotoxicity. The mutant diphtheria toxins CRM107 and CRM45 were used since their inability to bind cell receptors renders them ordinarily nontoxic. Their full cytotoxic potential, however, was restored via bispecific antibody-mediated delivery and release within low pH intracellular vesicles. Cytotoxicity was shown to be specific by blocking receptor sites and to be acidification-dependent by protection using NH4Cl to raise endosomal pH. Kinetics for inhibition of cellular protein synthesis was identical for native diphtheria toxin and the bispecific antibody-CRM107 combination. The rate of inhibition (t50 = 20 min) indicated that release of CRM107 from the antibody combining site was fast, and its toxic action was unimpeded by this delivery mechanism.

One of the earliest methods devised for the selective delivery of unmodified bioactive molecules into target cells used bispecific antibodies (1–3). By virtue of their dual specificity (4), these agents can noncovalently bind an effector moiety and simultaneously attach to specific antigenic sites on the cell membrane. Those initial studies used the plant toxin ricin or its enzymatically active A chain, whereas subsequent work used saporin (5) as a functional agent. Even though these complexes were internalized and ultimately killed target cells, neither the mechanism for antibody-toxin dissociation nor the intracellular compartment in which this release occurred was known.

Diphtheria toxin acts via a well defined intracellular pathway that culminates with an obligatory transfer of its toxic moiety from acidic endosomes to the cytosol (6, 7). That translocation is actuated by structural transitions in the toxin that are triggered in the pH 4.5–5.5 range (8) found within those organelles (9–11). Acid-induced conformational changes in the transmembrane domain of DT (12) are critical to the passage of its catalytic domain across vesicular membranes and into the cytosol, where it inactivates elongation factor 2 and kills the cell. These dynamic properties of DT have allowed us to design novel bispecific antibodies that not only accurately deliver toxin into target cells but also incorporate a precise mechanism for triggering its release upon entering endosomes, thereby exerting its full toxic potential.

We have selected murine monoclonal antibodies that tightly bind DT at neutral pH but spontaneously release the toxin as it undergoes a conformational change at pH 4.5–5.5 (see preceding paper (37)). Those antibodies were covalently linked to a second, cell-reactive antibody or receptor ligand to form hybrid molecules with dual specificity. The hybrid reagent carries antibody-bound toxin and specifically attaches to the surface of cells bearing a chosen target site. Toxin is released in its lethal form when the bispecific antibody-toxin complex is taken into cells and encounters the low pH in endosomes.

EXPERIMENTAL PROCEDURES

Materials—Na[125I] (17 Ci/mg) and L-[3,4,5-3H]leucine (140 Ci/mmol) were purchased from NEN Life Science Products. Iodogen was obtained from Pierce. Sephadex G-20, SPDP, high resolution Sephacryl S-300, phenyl-Sepharose, and XK16 columns were purchased from Pharmacia Biotech Inc. (Uppsala, Sweden).

Monoclonal Antibodies—The attributes of the TD3 monoclonal antibody directed against the human transferrin receptor have been reported (13). Monoclonal anti-DT antibodies were generated and characterized as described in the previous paper (37). A hybridoma cell line producing a human anti-DT monoclonal antibody designated F10 (14) was obtained from the American Type Culture Collection (Rockville, MD). Hybridoma clones were injected into pristane-primed BALB/c mice or Swiss nu/nu athymic mice (Taconic, Germantown, NY), ascites fluid was collected, and the 150-kDa anti-DT monoclonal antibody was isolated by (NH4)2SO4 precipitation and size fractionation on a high resolution Sephacryl S-300 column. The antibodies were >90% pure as judged by polyacrylamide gel electrophoresis. Immunoblot reactivity with the A versus B fragment of nicked DT was used to localize the region of antibody binding (15). Interaction with CRM mutant proteins and genetically engineered DT constructs helped to further characterize antibody specificity (see Table I).

Production of Bispecific Antibodies and Conjugates—A method for unidirectionally disulfide-linking two monoclonal antibodies has been described (3). The purified antibodies were each reacted with a 6-fold molar excess of SPDP, and the uncoupled reagent was removed by passage through a Sephade G-25 column equilibrated with 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5. This resulted in the addition of approximately 5 mol of SPDP/mol of antibody. To produce an appropriate bispecific antibody combination, the SPDP-substituted anti-transferrin receptor antibody was reduced with 50 mM dithiothreitol for 30 min, and the protein fraction was isolated by passage through a Sephadex G-25 column equilibrated with PBS. This thiolated antibody was then mixed with an SPDP-substituted anti-DT antibody, and the conjugates were allowed to react for 2 h at pH 7.0. For quantitative analysis of the conjugates, the bispecific antibody concentration was determined by measuring absorbance at 280 nm, and the antibody-toxin ratio was calculated based on the amount of delivered toxin.
Acid-sensitive Bispecific Antibodies

Isolation of Toxins—Diphtheria toxin was isolated (16) from a partially purified preparation purchased from Connaught Laboratories. The production and purification of ricin A chain from whole ricin was carried out as previously reported (17), (18). CRM107 and CRM45 (19–21) were obtained from cultures of the appropriate mutant strain of Corynebacterium diphtheriae as described earlier (19). Toxic was isolated from the culture supernatant either by (NH₄)₂SO₄ precipitation or by phenyl-Sepharose chromatography (22) and purified further as described (16). CRM107 was nicked by limited proteolytic cleavage with trypsin or urokinase (23).

Cell Lines—The human acute lymphoblastic leukemia CEM cell line and human colon adenocarcinoma LS 174T cell line were obtained from the American Type Culture Collection. The human mesothelioma H-Meso cell line (24) was used previously for cytotoxicity studies (13, 25). Cells were cultured at 37 °C with 5% CO₂. The resulting conjugate was purified by size exclusion chromatography.

Incorporation of [3H]leucine into cellular protein was measured by glass fiber filters using a Mash II cell harvester (Life Technologies, Inc.). No difference was seen when the bispecific antibody and its parent anti-DT were compared using 125I-DT binding titrations (37), indicating that they retained full activity.

The 7D3-CRM107 disulfide-linked covalent conjugate was formed in an analogous manner. The thiolated 7D3 monoclonal antibody was reacted with CRM107 that had been sparingly substituted with SPDP at neutral pH. The resulting conjugate was purified by size exclusion chromatography.

Hybrid Antibody-mediated Delivery of 125I-Toxin to Cellular Targets—Bispecific antibodies were formed by linking various anti-DT monoclonal antibodies (Table I) to an anti-human transferrin receptor monoclonal antibody designated 7D3. Dual specificity was verified by measuring the capacity of these reagents to attach simultaneously to transferrin receptors on the cell and bind 125I-toxin. The CEM cell line, which expresses high levels of transferrin receptor, served as a target, and two different protocols were followed to deliver 125I-DT via the hybrid antibodies. Cells were coated first with bispecific antibody by pretreatment at 2 °C for 30 min with an ensuing wash to remove unbound molecules. This surface-bound hybrid antibody with its empty toxin binding sites was allowed to capture the 125I-DT subsequently added. Alternatively, bispecific antibody and 125I-DT were precomplexed so that they could be bound to cell surface transferrin receptors as a single reagent. In each case, the use of anti-transferrin receptor/anti-DT bispecific antibodies resulted in the delivery of 5–10 times more 125I-DT to cells compared with their basal 125I-DT binding level (data not shown). This enhancement was transferrin receptor-specific, since preoccupying the target epitope with an excess of unmodified 7D3 blocked hybrid antibody attachment and subsequent 125I-DT binding.

Bispecific Antibody-mediated Cytotoxicity of CRM107—Hybrid antibody-mediated cytotoxicity was evaluated using two mutant forms of DT, CRM107 and CRM45, that have little or no inherent capacity for attaching to cells (27). CRM107 was bound to bispecific antibodies targeted against the transferrin receptors on a human mesothelioma cell line (H-Meso), and its inhibition of [3H]leucine incorporation into protein was measured (Table II). A 2-h incubation time was sufficient to obtain extensive cytotoxic effects. Whereas CRM107 alone is incapable of entering cells and inhibiting protein synthesis, it became a very effective and rapid-acting cytotoxin when used in combination with the anti-transferrin receptor/anti-DT bispecific antibody, 7D3/5E8.

This potent lethal action was dependent upon hybrid antibody-mediated delivery to transferrin receptors, since much less toxicity was observed when these target sites were blocked by including an excess of free anti-human transferrin receptor antibody (7D5) (Table II). The minor fraction of [3H]leucine incorporation that was not restored indicates that the blockade of receptors was not absolute. Separate experiments showed that the hybrid antibody was without effect in the absence of CRM107 and that mouse NS-1 cells, which lack the human transferrin receptor, were unaffected by toxin targeted by the 7D3-based bispecific antibody (data not shown).

The transferrin receptor was targeted because it normally cycles into acidic endosomes where Fe³⁺ release from transferrin is induced by the low pH (28). It was assumed that bispecific antibody-mediated cytotoxicity would likewise be dependent on the acidic environment of intracellular compartments, and this was demonstrated by adding NH₄Cl (29) to the cells. The weak base, which is known to raise vesicle pH, greatly reduced the ability of the bispecific antibody-CRM107

### Table I

| Antibody | Epitope domain | Origin | Acid release
|----------|----------------|--------|---------------|
| 5D5      | Transmembrane  | Mouse  | Yes           |
| 4B7      | Catalytic      | Mouse  | Yes           |
| 5F5      | Catalytic      | Mouse  | No            |
| 1F3      | Receptor binding Mouse | Yes | |
| 5E8      | Receptor binding Mouse | Yes | |
| F10      | Receptor binding Human | No | |

* Release from DT was tested in the pH 4.0–5.5 range (37).

RESULTS

Hybrid Antibody-mediated Delivery of 125I-Toxin to Cellular Targets—Bispecific antibodies were formed by linking various anti-DT monoclonal antibodies (Table I) to an anti-human transferrin receptor monoclonal antibody designated 7D3. Dual specificity was verified by measuring the capacity of these reagents to attach simultaneously to transferrin receptors on the cell and bind 125I-toxin. The CEM cell line, which expresses high levels of transferrin receptor, served as a target, and two different protocols were followed to deliver 125I-DT via the hybrid antibodies. Cells were coated first with bispecific antibody by pretreatment at 2 °C for 30 min with an ensuing wash to remove unbound molecules. This surface-bound hybrid antibody with its empty toxin binding sites was allowed to capture the 125I-DT subsequently added. Alternatively, bispecific antibody and 125I-DT were precomplexed so that they could be bound to cell surface transferrin receptors as a single reagent. In each case, the use of anti-transferrin receptor/anti-DT bispecific antibodies resulted in the delivery of 5–10 times more 125I-DT to cells compared with their basal 125I-DT binding level (data not shown). This enhancement was transferrin receptor-specific, since preoccupying the target epitope with an excess of unmodified 7D3 blocked hybrid antibody attachment and subsequent 125I-DT binding.

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combination to kill H-Meso cells (data not shown). Low vesicular pH is essential for action, since it both triggers release of CRM107 from the antibody and induces translocation of toxin into the cytosol, where it enzymatically inactivates elongation factor 2.

Dose response curves were generated to better characterize the multicomponent system. H-Meso cells were treated with a fixed high level of the bifunctional antibody, 7D3/5E8, plus increasing amounts of CRM107 (Fig. 1A). Maximal cytotoxicity was attained as the ratio of CRM molecules to toxin binding sites approached 2:1. Likewise, cells were exposed to a set high concentration of CRM107 plus varying amounts of the bispecific antibody. Cytotoxicity on both of the cell lines tested reached a maximum level when 7D3/5E8 was at $10^{-8}$ M or higher (Fig. 1B).

The transferrin receptor-directed hybrid antibody-CRM107 complex was also assayed on human colon adenocarcinoma cells to determine if the same high cytotoxic potency found for the H-Meso line extended to other malignant cell types. The combined action of CRM107 plus hybrid at $10^{-9}$ M produced extensive killing of these cells within 2 h, and its potency was comparable to $10^{-7}$ M native DT (Table III). These results indicated that bispecific antibody delivery not only rendered CRM107 cytotoxic to cells but also suggested that its entry via the transferrin pathway was as efficient as DT uptake by its usual mechanism.

**Kinetics of Cytotoxicity**—A fundamental premise underlying the acid-triggered bispecific antibody carrier concept is that the mechanism of toxin action proceeds normally after specific targeting has been achieved. The kinetics for inhibition of protein synthesis gives a critical measure of toxin efficiency by monitoring how rapidly it gains access to and inactivates elongation factor 2 in the cytosol. This parameter was therefore used to evaluate bispecific antibody-delivered CRM107 (Fig. 2).

The time course of protein synthesis inhibition in H-Meso cells was monitored following the addition of native DT alone or the 7D3/5E8 bispecific antibody complexed with either CRM107 or nicked CRM107. Each of these agents gave a comparable kinetics pattern, which was characterized by a 15-min lag period followed by a rapid first order inactivation phase, with $t_{1/2} = 20$ min (Fig. 2). The fact that hybrid antibody-delivered CRM107 killed cells as rapidly as native DT suggests that its release from the antibody combining site was unimpeed and that translocation into the cytosol proceeded efficiently. Proteolytic cleavage of the bispecific antibody-delivered CRM107 was apparently not rate-limiting, since prior nicking of the toxin had little effect on its rate of action (Fig. 2). Similar biphasic kinetics patterns were given by the 7D3/3E1 and 7D3/4B7 hybrid antibodies plus CRM107, with a $t_{1/2} = 24$ and 36 min, respectively. The 5E8 and 3E1 antibodies recognized an epitope localized on the receptor binding domain of DT, whereas 4B7 bound to the catalytic domain (Table I).
Acid-sensitive Bispecific Antibodies

Comparison of Covalently-linked and Bispecific Antibody-delivered CRM107—A covalently-coupled anti-transferrin receptor-CRM107 conjugate (7D3-CRM107) was constructed by standard disulfide linkage methods. Dose response curves were generated by treating CEM cells for 16 h at 37 °C with varying concentrations of either native DT, the 7D3-CRM107 covalent conjugate, or the 7D3/5E8 bispecific antibody in the presence of 10^{-7} M CRM107 (Fig. 3). CEM cells are not particularly sensitive to DT as reflected by the IC_{50} = 2 \times 10^{-9} M obtained with native toxin and the prolonged incubation time required for cytotoxicity. The transferrin receptor-directed 7D3-CRM107 disulfide conjugate was slightly more effective, giving an IC_{50} = 1 \times 10^{-9} M. In contrast, hybrid-delivered CRM107 (IC_{50} = 4 \times 10^{-12} M) was 250-fold more potent than the covalent conjugate, based upon the concentration of hybrid antibody added. Neither the 7D3/5E8 bispecific antibody alone nor CRM107 alone at 1 \times 10^{-7} M had an effect upon the cells (Fig. 3). When tested on H-Meso cells, the IC_{50} values for the 7D3-CRM107 disulfide conjugate and 7D3/5E8-delivered CRM were 2.5 \times 10^{-9} and 7 \times 10^{-12} M, respectively, a 360-fold differential. These results indicate that covalent coupling actually impedes toxin action since the disulfide-linked 7D3-CRM107 conjugate was much less potent than the corresponding 7D3/5E8 hybrid antibody-delivered CRM107. This observation may have important implications for the relative therapeutic effectiveness of these two types of anti-tumor agents when tested in animal model systems.

DISCUSSION

We originally designed hybrid antibodies with dual specificity to deliver sensitive bioactive molecules into target cells in a nondestructive manner. These reagents used an antibody to hold the molecule reversibly within its combining site while the second antibody or ligand component accurately targeted the complex to selected cells (1–3). This delivery method circumvents the potential steric inactivation of delicate molecules that may result from coupling to a carrier moiety by covalent link-

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**Fig. 2. Kinetics of cellular protein synthesis inhibition.** H-Meso cells were incubated for the designated intervals after the addition of 10^{-5} M native DT alone (○) or 10^{-4} M of the 7D3/5E8 bispecific antibody complexed with either CRM107 (■) or nicked CRM107 (□). Inhibition of [3H]leucine incorporation was measured and expressed as the percent of protein synthesis compared with untreated control cells. When protein synthesis was reduced to <10% that of the control level, the values became less reliable and were not weighted as strongly.

**TABLE IV**

Cytotoxicity of acid-sensitive versus acid-insensitive hybrid antibody-delivered CRM107

| Additions | Incubation time | [3H]Leucine incorporation | Inhibition |
|-----------|----------------|--------------------------|------------|
| None      | 3              | 73,225                    | 82         |
| + CRM107  | 24             | 71,445                    | 99         |
| + 7D3/5E8 + CRM107 | 13,025 | 82                        |
| + 7D3/F10 + CRM107 | 82,360 | 0                        |

**TABLE V**

Bispecific antibody-mediated cytotoxicity of CRM45

| Additions | [3H]Leucine incorporation | Inhibition |
|-----------|--------------------------|------------|
| None      | 111,265                  | 8          |
| + CRM45   | 105,680                  | 5          |
| + 7D3/5D5 + CRM45 | 6,030 | 95                      |
| + 7D3/5F5 + CRM45 | 102,865 | 8                      |

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Acid-sensitive Bispecific Antibodies

The acid-triggered bispecific antibodies were shown to mediate the cytotoxicity of CRM107 and CRM45, which are ordinarily inactive because they cannot bind to cells. These bispecific antibody-delivered CRMs killed most of the target cells within 2 h. When examined in more detail, the kinetics for inhibition of cellular protein synthesis was identical for the bispecific antibody-CRM107 combination and native DT, showing a $t_{1/2}$ of 20 min for the rapid phase. This indicated that CRM released quickly from the antibody carrier and that its transmembrane domain facilitated the translocation of its enzymatic domain into the cytosol in a normal manner. The effectiveness of the CRM45 deletion mutant is especially pertinent since this protein consists solely of those two domains. Previous reports showed that truncated molecules such as CRM45 did not possess full cytotoxic activity when covalently linked to monoclonal antibody or other binding moieties (27, 30, 31). Such attenuation might be caused by steric encroachment of the closely coupled antibody on the translocation domain since our results revealed no inherent translocation deficiency in the CRM45 molecule.

Target cells were protected from the toxic action of hybrid antibody-delivered CRM by using NH$_4$Cl to raise endosomal pH. This perturbation precluded both the acid-triggered release of toxin from the antibody combining site and the pH-induced translocation step needed for efficient cytotoxicity. Moreover, CRM107 delivered into cells using acid-insensitive bispecific antibodies was inactive, even after prolonged incubation. These antibodies bind to toxin epitopes that do not undergo a conformational change at low pH, and the toxin therefore remains bound inside the endosome. Failure to release within endosomal vesicles may result in the re-routing of toxin to ensuing compartments like the golgi or to lysosomes where it is destroyed. These combined neutralization and acid-stable antibody results confirmed that CRM toxins must release from the antibody in acidified endosomes to function properly. Conversely, the system provides strong evidence for the acid-induced breakup of certain specific antigen-antibody complexes within the low pH vesicles of living cells.

Treatment of target cells with hybrid antibodies carrying ricin A chain instead of CRM107 showed an extremely delayed cytotoxic response that was unaffected by neutralization of endosomal pH by NH$_4$Cl (data not shown). This slow action was in sharp contrast to the rapid acting, pH-sensitive, DT-based system and prevailed even though delivery via the transferrin pathway in the same cells was mediated by an identical targeting antibody. Unlike the direct pH-dependent endosomal pathway taken by DT, many toxins like ricin, Shiga toxin, and *Pseudomonas* exotoxin apparently follow more intricate and time consuming pathways before reaching their targets in the cytosol. The point of egress for these toxins may be the endoplasmic reticulum or Golgi apparatus rather than the

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**FIG. 3. Cytotoxicity dose response curves.** Transferrin receptor positive CEM cells were incubated for 16 h with the designated concentrations of the 7D3/5E8 bispecific antibody plus $10^{-7}$ M CRM107 (●), the 7D3-CRM107 disulfide-linked conjugate (□), native DT (○), CRM107 alone (■), or the 7D3/5E8 bispecific antibody alone (▲). The cells were then pulse-labeled with [H]$^3$H]leucine for 30 min, and the amount of incorporation into protein was compared with untreated control cells.

**FIG. 4. Delivery and acid-triggered release of toxin using bispecific antibodies.** Diphtheria toxin and CRMs undergo a conformational change at pH ≤ 5. Acid-sensitive monoclonal antibodies that recognize the epitopes involved in this transition were disulfide-linked to targeting antibodies that attach to cell surface sites. The resulting bispecific antibody bound the CRM toxin at neutral pH and selectively delivered it to the cell membrane where the complex was taken into cells by endocytosis. Acid conditions within the endosome triggered release of CRM from the antibody and induced its translocation across the vesicle membrane. Once in the cytosol, the catalytic domain of the toxin killed cells by inactivating elongation factor 2 and curtailing protein synthesis.
Acid-sensitive Bispecific Antibodies

The bispecific antibody approach is very flexible since antibodies or ligands to virtually any membrane site can be easily coupled with the toxin-bearing, pH-sensitive antibody to form a variety of highly specific delivery agents. In addition to using the anti-transferrin receptor monoclonal antibody to target cells, we have also disulfide-linked diferric transferrin (18) to an acid-releasable anti-DT antibody, and this bifunctional construct also mediated CRM cytotoxicity via the same internalization pathway (data not shown). Endocytotic cycling though an acid compartment is required for cytotoxic effectiveness using these DT-based systems, but that condition seems to be the rule for surface receptor sites rather than the exception.

Anthrax toxin operates through an acid-dependent mechanism that is similar to that used by DT (34). One component of anthrax toxin undergoes a pH-induced conformational change and oligomerization to assist the translocation of its effector portion out of the endosome and into the cytosol (35). Thus this toxin or its components might also be targeted to cells and released inside by using an acid-triggered bispecific antibody approach analogous to the one developed for DT.

Whereas the present study utilized bispecific antibodies formed by linking two whole antibodies, we have also made exclusively heterobispecific F(\text{ab}')_2 hybrid antibodies (36) using the same targeting and acid-sensitive monoclonal antibodies described in this paper. These smaller F(\text{ab}')_2 reagents (\sim 100 kDa) have proven to be slightly superior delivery vehicles on a molar basis compared with the bivalent whole antibody constructs. Their effectiveness dispels the notion that cross-linking of two surface receptors is necessary for the cellular uptake of bispecific antibody-delivered toxins, since these reagents are monovalent for both the toxin and the cell surface epitope. The production of analogous bispecific single chain Fv reagents (\sim 50 kDa) for toxin delivery is feasible, and their use for therapeutic purposes might offer an advantage.

By providing both the selective delivery to target cells and a pH-controlled intracellular release of biological agents, acid-triggered bispecific antibodies might offer some advantages for therapeutic use. Moreover, the hybrid antibody and active ligand could be administered either as separate components or as a single preloaded complex. However, to maintain a high occupancy of antibody combining sites in vivo, either high affinity antibodies or elevated levels of ligand would have to be used.

The ability to deliver the individual functional domains of DT independently into the same cell using this bispecific antibody delivery system has opened an interesting prospect. The catalytic, transmembrane, and receptor binding domains of DT can be produced by genetic engineering, and separate acid-releasable hybrid antibodies that bind to these constituent domains have been produced. Therefore, we intend to test if those toxin components can cooperate to produce cytotoxicity when they are delivered into the cell as separate entities and are released within the same acidified endosome.

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