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Mutations in Diphtheria Toxin Separate Binding from Entry and Amplify Immunotoxin Selectivity

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Monoclonal antibodies linked to toxic proteins (immunotoxins) can selectively kill some tumor cells in vitro and in vivo. However, reagents that combine the full potency of the native toxins with the high degree of cell type selectivity of monoclonal antibodies have not previously been designed. Two heretofore inseparable activities on one polypeptide chain of diphtheria toxin and ricin account for the failure to construct optimal reagents. The B chains (i) facilitate entry of the A chain to the cytosol, which allows immunotoxins to efficiently kill target cells, and (ii) bind to receptors present on most cells, which imparts to immunotoxins a large degree of non–target cell toxicity. This report identifies point mutations in the B polypeptide chain of diphtheria toxin that block binding but allow cytosol entry. Three mutants of diphtheria toxin have 1/1,000 to 1/10,000 the toxicity and 1/100 to 1/8,000 the binding activity of diphtheria toxin. Linking of either of two of the inactivated mutant toxins (CRM103, Phe^{508}; CRM107, Phe^{390}, Phe^{525}) to a monoclonal antibody specific for human T cells reconstitutes full target-cell toxicity—indistinguishable from that of the native toxin linked to the same antibody—without restoring non–target cell toxicity. This separation of the entry function from the binding function generates a uniquely potent and cell type–specific immunotoxin that retains full diphtheria toxin toxicity, yet is four to five orders of magnitude less toxic than the native toxin is to nontarget cells.

DIPHTHERIA TOXIN (DT) AND RICIN are potent toxins composed of two disulfide-linked polypeptide chains (*1*). The B chains bind the toxin to the cell surface and facilitate transport of the A chain to the cytosol. The A chains catalytically inhibit protein synthesis, and a single molecule of either DT A chain (*2*) or ricin A chain (*3*) in the cytosol is sufficient to kill a cell. The combination of these three activities—binding, translocation, and catalysis—produces the extreme potency of these proteins.

Monoclonal antibodies specific for tumor-cell surface antigens have been linked to toxins or toxin subunits to generate a new class of therapeutic drugs called immunotoxins (*4*). Toxin A chains linked to monoclonal antibodies show a high degree of cell type selectivity in vitro but little toxicity to solid tumors in vivo (*5*). This low toxicity may be due, in part, to slow transport of the A chain to the cytosol (*6, 7*).

Target cell toxicity of immunotoxins can be increased by including the toxin B chain in the antibody-toxin complex (*8*) or by adding it separately (*7, 9*). To achieve maximal in vitro target cell selectivity with immunotoxins containing intact ricin, lactose must be added to the medium to block non–target cell binding and toxicity of the immunotoxin via the ricin B chain. This approach is feasible in those clinical settings, such as bone marrow transplantation (*10*), where the target cell population can be incubated in vitro in the presence of lactose. Without blockage of the B-chain binding domain, however, whole-toxin conjugates have a high degree of non–target cell toxicity, thereby reducing their usefulness in vivo to that of A-chain immunotoxins (*11*).

Construction of reagents that combine the potency of intact toxin conjugates with the cell type selectivity of toxin A-chain conjugates may be possible if the binding site on the toxin B chain could be irreversibly blocked. Covalent and noncovalent chemical modifications that block the binding activity of ricin intracellularly also block its entry function, which suggests that the binding and translocation functions may be inseparable (*12*); however, inactivation of ricin binding by steric hindrance or by oxidation blocks non–target cell toxicity more than target cell toxicity (*13*).

Previously, domain deletion was used in an unsuccessful attempt to separate the translocation and the binding functions of the B chain of DT (*14*). Immunotoxins made with DT A chain, intact DT, and a

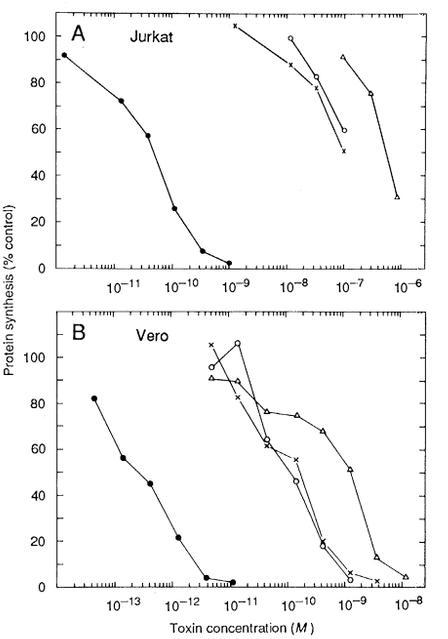

Fig. 1. Toxicity of CRM102, CRM103, CRM107, and native DT to Jurkat cells (**A**) and Vero cells (**B**). Protein synthesis was assayed by incubating 5×10^4 Jurkat cells in 100 μl of leucine-free RPMI 1640 medium containing 2% fetal bovine serum (FBS) in 96-well microtiter plates. DT (●), CRM102 (x), CRM103 (○), or CRM107 (△) was added in 11 μl of buffer and incubated with cells for 16 hours at 37°C. Cells were then pulsed with 20 μl of phosphate-buffered saline containing 0.1 μCi of [^{14}C]leucine, incubated for 1 hour at 37°C, harvested onto glass fiber filters by means of a PHD cell harvester (Cambridge Technology), washed with water, dried, and counted. The results are expressed as a percentage of the [^{14}C]leucine incorporation in mock-treated control cultures.

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cloned fragment of DT (MspSA) that lacks the COOH-terminal 17-kD region of the B subunit were compared. The intact DT conjugate was 100 times as toxic as the MspSA conjugate, which, in turn, was 100 times as toxic as the DT A-chain conjugate. The COOH-terminal 17-kD region, which contains the cell surface binding site (*15*), therefore potentiates immunotoxin activity 100-fold. It was not possible to determine whether this COOH-terminal translocation activity was distinct from the binding activity. Similarly, CRM45 of DT was linked to the hormone MSH, and PE40 of pseudomonas toxin was linked to the growth factor TGFα, in attempts to eliminate the toxin binding site and maintain the B-chain entry function (*16*). In this report we identify point mutations in the COOH-terminal region that locate the DT binding site and that enable full separation of the binding and translocation functions of the toxin.

Laird and Groman mutagenized *Corynebacterium* with nitrosoguanidine and ultraviolet irradiation and isolated several classes of mutants within the DT structural gene (*17*). Leppla and Laird further characterized several of the mutant proteins and found that three of them (CRM102, CRM103, and CRM107) retained full enzymatic activity but had defective receptor binding (*18*).

A comparison of the toxicities of these three mutants with that of native DT on two cell types is shown in Fig. 1. Vero cells have a higher number of DT receptors than do Jurkat cells and are thus more sensitive to DT inhibition of protein synthesis than are Jurkat cells. CRM102 and CRM103 are 1/1,000 as toxic as native DT is, and CRM107 is 1/10,000 as toxic as native DT is, to both Vero cells and Jurkat cells (*19*).

The binding activity of native DT and the three CRM mutants to Vero cells is shown in Fig. 2. Whereas most cell types, including lymphoid cells such as Jurkat, have undetectable levels of DT receptors (*20*), Vero cells contain 10^5 DT receptors per cell and have been used extensively to study DT binding (*20*). At 4°C the affinity of both CRM102 and CRM103 is 1/100 that of native DT, and the affinity of CRM107 is 1/8000 that of native DT (*21*).

The reduced affinity correlates with the reduced toxicity for CRM107 but differs by a factor of 10 for CRM102 and CRM103. Binding was determined after 6 hours at 4°C, while toxicity was determined after 24 hours at 37°C. The discrepancy between binding and toxicity for CRM102 and CRM103 may reflect differences in temperature and time in the two assays. Binding cannot be determined at 37°C since energy inhibitors commonly used to block internalization decrease the number of surface DT receptors (*20*). Alternatively, the mutation or mutations within CRM102 and CRM103 may inhibit toxin activities other than binding that may account for the tenfold difference between toxicity and binding.

The location of the amino acid changes within the B chain for each of the three CRMs is shown in Fig. 3. CRM103 contains a single mutation at position 508 (Ser → Phe). CRM102 contains a similar mutation at position 508 but has an additional mutation at position 308 (Pro → Ser). CRM107 contains two mutations, one at position 390 (Leu → Phe) and one at position 525 (Ser → Phe). That CRM102 has two mutations, whereas CRM103 contains only one, indicates that the two mutants are independent isolates. The presence of multiple GC-AT transitions is consistent with nitrosoguanidine-induced mutagenesis.

The binding affinity of CRM103 and CRM102 that is 1/100 that of DT demonstrates that the serine at position 508 is important for toxin binding. The conservative change in CRM107 at position 390 (Leu → Phe) suggests that the alteration at position 525 causes the 8000-fold difference in binding activity, such that CRM107 is less active than DT. The mutations at positions 508 and 525 are consistent with data that suggest that the DT binding domain lies within the carboxyl 17-kD portion of the molecule (*15*). Both mutations exchange a phenylalanine for a serine.

The relation of binding to translocation in DT was examined by linking each of the CRMs and native DT to a new binding domain, the monoclonal antibody UCHT1, specific for the T3 antigen on human T cells. Unlike the unconjugated CRMs, all three CRM immunotoxins are highly toxic (Fig. 4). Excess antibody blocks toxicity, demonstrating that the toxicity is antibody-mediated. The immunotoxins prepared with CRM103 and CRM107 are as toxic as the immunotoxin prepared with native DT, whereas the immunotoxin prepared from CRM102 is less toxic by a factor of approximately 10. The tenfold decrease in UCHT1-CRM102 toxicity relative to UCHT1-CRM103, despite identical binding activity of CRM102 and CRM103, suggests that the amino acid at position 308 contributes to the translocation activity of DT. That the conjugates prepared with CRM103 and CRM107 are as toxic as conjugates prepared with native DT indicates that binding of the toxin to its receptor is not necessary for

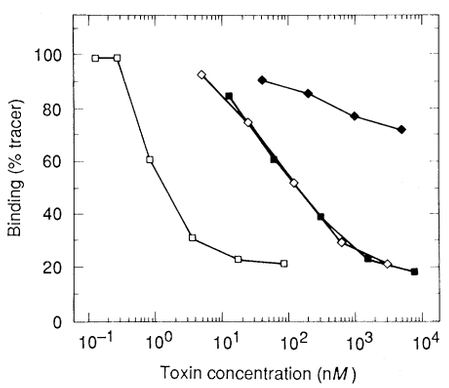

Fig. 2. Binding of native DT, CRM102, CRM103, and CRM107 to Vero cells. Tracer ^{125}I-labeled DT binding was determined by competition with cold DT (□), CRM102 (■), CRM103 (◇), and CRM107 (◆). DT was labeled with Biobeads to 7×10^6 cpm/μg, as reported previously (*25*). Vero cells, plated the previous day at 5×10^5 cells per milliliter per well in Costar 24-well plates, were incubated in 150 μl of Dulbecco's minimum essential medium and 10% FBS and 25 m*M* Hepes (*p*H 7.0), with ^{125}I-labeled DT (8 ng/ml) and appropriate concentrations of DT and CRMs. After incubating 6.5 hours at 4°C, cells were washed four times in complete medium, solubilized in 0.1*N* NaOH, and counted. Tracer binding varied between 900 and 1500 cpm, depending on the experiment. No nonspecific binding was subtracted.

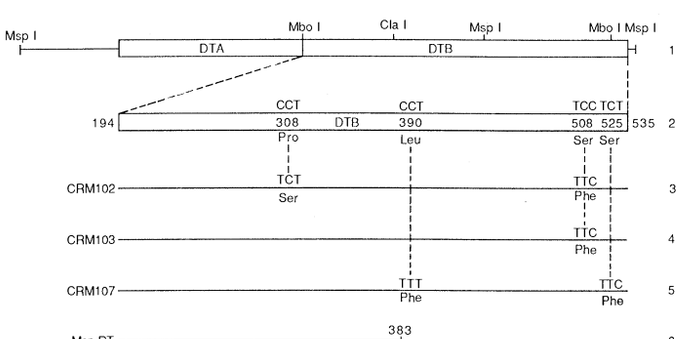

Fig. 3. Location of the CRM point mutations within the DT structural gene (*26*). (Line 1) Restriction map of the DT structural gene, indicating the location of the sites used for sequencing. (Line 2) Expansion of the B-chain structural region, indicating the native amino acid and DNA sequence corresponding to the point mutations found within the CRMs. Mutations found within the B chain of CRM102 (line 3), CRM103 (line 4), and CRM107 (line 5) are shown. (Line 6) The end of the MspRT clone is shown as described (*14*). The sequences were obtained by cloning the two Mbo I–Cla I fragments into M13MP and M13MP19 and sequencing by the method of Sanger (*27*) or by cloning the two Msp I fragments into pBR322 and sequencing by the method of Maxam and Gilbert (*28*).

efficient translocation of the toxin-A fragment to the cytosol. Therefore, the DT binding and translocation functions can be separated.

Native DT and UCHT1-DT inhibit Jurkat cell protein synthesis 50% at $3 \times 10^{-11} M$ (Figs. 1 and 4). The toxicity of UCHT1-DT to T3-bearing cells is 100 times that to other cells (*22*), and the selectivity exists solely because cross-linking DT to antibody inhibits DT-mediated toxicity 100-fold. The mutant toxins CRM102, CRM103, and CRM107 inhibit Jurkat cell protein synthesis 50% at $1 \times 10^{-7} M$ to $4 \times 10^{-6} M$ (Fig. 1), whereas the UCHT1-CRM immunotoxins act at $3 \times 10^{-11} M$ to $3 \times 10^{-10} M$ (Fig. 4). This 1,000- to 10,000-fold difference between the CRMs and the UCHT1-CRMs in the concentration required to inhibit protein synthesis represents an increase from three to four orders of magnitude in CRM-immunotoxin selectivity over the native DT immunotoxin.

The toxicities of the different immunotoxins were compared on nontarget Vero cells, which lack antibody binding sites but express a high number of DT cell surface binding sites. UCHT1-DT inhibits Vero protein synthesis 90% at $6 \times 10^{-10} M$, because of toxicity via the DT binding site. In contrast, the three CRM immunotoxins had no effect on protein synthesis at this concentration. To determine the full extent of nontarget cell toxicity of the binding site–inactivated immunotoxins, large amounts of a monoclonal antibody–CRM107 conjugate were made, purified, and assayed on Vero cells. A full dose-response curve (Fig. 5) shows that the CRM107 immunotoxin is 1/2,000 times as toxic as the native DT immunotoxin and is 1/200,000 times as toxic as DT. Because the CRM107 immunotoxins are as toxic to target cells as native DT (Figs. 1 and 4), the selectivity of CRM107 immunotoxins to any one cell type may be as great as 200,000-fold. Thus, the loss of toxicity of the CRMs is exhibited also by the CRM immunotoxins on nontarget cells. Preliminary studies in vivo show that CRM107 is at least 1/100 as toxic to guinea pigs as DT. A large decrease in nontarget cell toxicity of CRM107 immunotoxins occurs in vitro and in vivo.

Immunotoxins made with antibodies and whole toxins that are genetically altered in their binding domain have several advantages over antibody-toxin A-chain conjugates. First, as shown with DT, the B-chain translocation activity can be utilized in the absence of its binding function to increase the potency of CRM107 conjugates 10,000-fold over that of A-chain conjugates (*14*). Furthermore, recent data suggest that the disulfide linkage between various A chains

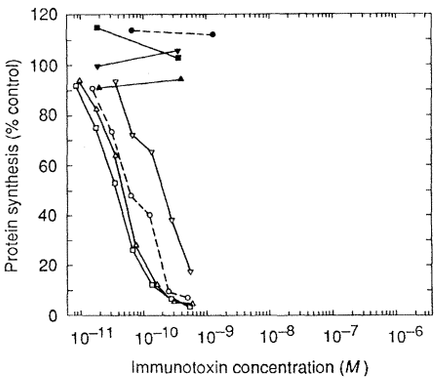

Fig. 4. Comparison of the toxicities of immunotoxins made by conjugating UCHT1 with CRM102, CRM103, CRM107, and native DT. The antibody was linked to the toxins via a thioether bond as described previously (*22*). Immunotoxins were separated from unconjugated antibody and toxin by gel filtration on a TSK-3000 high-performance liquid chromatography column. The immunotoxin peak was collected, and toxicity was evaluated with the protein synthesis assay described in the legend to Fig. 1. UCHT1-DT (○), UCHT1-CRM102 (▽), UCHT1-CRM103 (△), and UCTH1-CRM107 (□) were incubated with 5×10^4 Jurkat cells for 16 hours, followed by a 1-hour pulse with [^{14}C]leucine. Incubation with excess free UCHT1 (100 μg/ml) blocked toxicity (closed symbols).

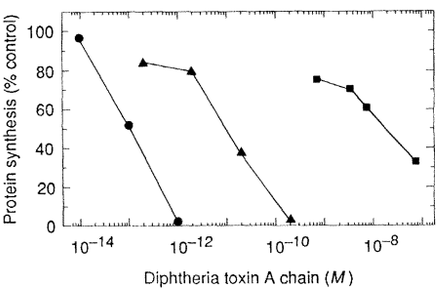

Fig. 5. Non–target cell toxicity of a CRM107 immunotoxin and a native DT immunotoxin compared to DT toxicity. Immunotoxins were made and purified as in the legend to Fig. 4 with the control monoclonal antibody 454A12 that does not react with Vero cells. Vero cells were incubated for 24 hours with native DT, the 454A12-DT immunotoxin, and the 454A12-CRM107 immunotoxin then pulsed with [^{14}C]leucine for 2 hours and processed as described in the legend to Fig. 1. DT (●), DT immunotoxin (▲), CRM107 immunotoxin (■).

and antibody is unstable in vivo (*23*). Reduction of the disulfide linkage leads to rapid loss of immunotoxin in vivo and the release of free antibody that can bind to the tumor cells and compete with intact immunotoxins. Use of whole toxins permits construction of noncleavable thioether linkages between toxin and antibody. Finally, intact toxins are less susceptible to proteolytic inactivation than toxin-A fragments (*24*) and may survive longer in vivo.

Intact DT contains three activities: (i) enzymatic adenosine 5′-diphosphate (ADP)–ribosylation activity, located in fragment A; (ii) cell-surface receptor binding; and (iii) cytosol translocation activity, located in fragment B. This report shows that the binding activity can be separated from the translocation activity and a greatly improved cell type–specific toxin can be made. The new immunotoxins have full A-chain activity and full B-chain translocation activity, but they lack native DT binding (have 1/100 to 1/8000 the binding activity) and possess a new binding domain covalently attached. This new type of immunotoxin combines the full killing efficiency of the native toxin with the high specificity for cell type of monoclonal antibodies.

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Family Study of Platelet Membrane Fluidity in Alzheimer's Disease

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The fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in labeled platelet membranes, an index of membrane fluidity, identifies a prominent subgroup of patients with Alzheimer's disease who manifest distinct clinical features. In a family study, the prevalence of this platelet membrane abnormality was 3.2 to 11.5 times higher in asymptomatic, first-degree relatives of probands with Alzheimer's disease than in neurologically healthy control subjects chosen without regard to family history of dementia. The pattern of the platelet membrane abnormality within families was consistent with that of a fully penetrant autosomal dominant trait. Thus, this abnormality of platelet membranes may be an inherited factor that is related to the development of Alzheimer's disease.

MOUNTING EVIDENCE SUGGESTS that Alzheimer's disease is associated with pathologic changes in cells outside the central nervous system (*1, 2*). Several of the abnormalities described in nonneural cells reflect an alteration in cell membrane structure or function. Among these is an increase in platelet membrane fluidity, as revealed by a reduction in the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in labeled membranes. This method provides a measurement of the degree to which the rotation of DPH molecules in labeled membranes is hindered and, therefore, provides an index that is inversely related to membrane fluidity (*3*). An increase in platelet membrane fluidity associated with Alzheimer's disease has been observed by us in blind studies of patients from Boston (*4, 5*) and Pittsburgh (*6, 7*), and has been replicated by others in London (*8*). Abnormalities of cell membrane composition and structure have also been found in brain tissue obtained at autopsy from patients who died with confirmed Alzheimer's disease. These abnormalities include changes in brain phospholipid metabolism as revealed by nuclear magnetic resonance spectroscopy (*9*), disordering of cortical myelin as indicated by x-ray diffraction (*10*), and an alteration in the molecular dynamics of hippocampal membranes as reflected by fluorescence spectroscopy (*11*).

Initial assessments of the specificity of this platelet membrane alteration in other neuropsychiatric disorders have been promising. The increase in platelet membrane fluidity associated with Alzheimer's disease was not found in platelets from patients with depression, a common cause of reversible dementia in the elderly (*7*), in patients with mania, which may also be accompanied by a secondary dementia (*12*), or in patients with multi-infarct dementia (*8*).

A cutoff point for DPH anisotropy of 0.1920 at 37°C (90th percentile for healthy elderly controls) (Fig. 1) segregates patients with Alzheimer's disease into two clinical subtypes. As a group, patients with increased platelet membrane fluidity (DPH anisotropy less than 0.1920) suffer from an earlier symptomatic onset and have a more rapidly progressive course. A family history of dementia also appears to be a more common feature of patients in this group. (*7*).

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At the cellular level, several lines of evidence suggest that the increase in platelet membrane fluidity associated with Alzheimer's disease results from an accumulation of internal membranes rather than a generalized abnormality of cell membranes (*13*). Ultrastructural studies have revealed an excess of atypical cells containing an overabundant system of trabeculated cisternae bounded by smooth membrane in platelet preparations from patients with Alzheimer's disease. Menashi *et al.* have reported that internal membranes exhibit higher membrane fluidity than external platelet membranes, as reflected by DPH anisotropy (*14*). Therefore, a relative increase in internal membranes may account for the increase in platelet membrane fluidity associated with Alzheimer's disease. In support of this hypothesis, Cohen *et al.* found that platelets that exhibit increased membrane fluidity also manifest a reduction in the cholesterol: phospholipid ratio that could be accounted for by an approximate doubling of the usual mass of internal membranes per cell (*15*). In addition, when intact platelets from patients with Alzheimer's disease are labeled with DPH, a process that preferentially labels external membrane, they fail to exhibit an alteration in fluorescence anisotropy (*13*). Moreover, erythrocyte ghosts, which lack internal membranes, also fail to exhibit an alteration in membrane fluidity as reflected by fluorescence (*13*) or electron spin resonance spectroscopy (*16*). This evidence suggests that the increase in platelet membrane fluidity associated with Alzheimer's disease may result from a dysregulation of platelet membrane biogenesis or turnover.

Our family study was conducted to determine whether increased platelet membrane fluidity aggregates in the families of patients with Alzheimer's disease and, if so, whether this platelet abnormality selectively runs in the families of probands who themselves exhibit the abnormality. A series of 23 patients with probable Alzheimer's disease who had at least one available first-degree relative living in the Pittsburgh area were chosen as probands from the large cohort of patients described in Fig. 1. The diagnosis of probable Alzheimer's disease was made according to currently accepted clinical criteria as applied conjointly by Board-certified neurologists and psychiatrists (*17*). An additional 15 patients with Alzheimer's disease that had been confirmed by autopsy, from whom platelet membranes could not be obtained but who had at least one available first-degree relative, also served as probands. Neuropathologic diagnoses were made by Board-certified neuropathologists according to current consensus criteria (*18*). The participation of all neurologically healthy first-