An Anti-CD3 Single-chain Immunotoxin with a Truncated Diphtheria Toxin Avoids Inhibition by Pre-existing Antibodies in Human Blood*

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Diphtheria toxin (DT) is often used in the construction of immunotoxins. One potential problem using DT-based immunotoxins is the pre-existing anti-DT antibodies present in human blood due to vaccination. The present study examined the effect of human serum with pre-existing anti-DT antibodies on the toxicity of UCHT1-CRM9, an immunotoxin directed against CD3 molecules on T-lymphocytes. Sera with detectable anti-DT antibodies at 1:100 or greater dilutions inhibited the immunotoxin toxicity. Experiments with radiolabeled UCHT1-CRM9 indicate that anti-DT antibodies partially block its binding to the cell surface as well as inhibit the translocation from the endosome to the cytosol. The inhibitory effect could be adsorbed using a full-length DT mutant or B-subfragment. A C-terminal truncation mutant could not adsorb the inhibitory effect, suggesting that the last 150 amino acids contain the epitope(s) recognized by the inhibitory antibodies. Therefore, an anti-CD3 single-chain immunotoxin, sFv-DT390, was made with a truncated DT. The IC₅₀ of sFv-DT390 was 4.8 × 10⁻¹¹ M, 1/16 the potency of the divalent UCHT1-CRM9. More importantly, sFv-DT390 toxicity was only slightly affected by the anti-DT antibodies in human sera.

Mutated full-length and truncated diphtheria toxin (DT) molecules are used for making immunotoxins. These immunotoxins show strong cytotoxic effects to their target cells, and some of them have already been used in clinical trials (1–7). Previously, our laboratory constructed an immunotoxin directed against the CD3ε molecule of the T-cell receptor complex, a pan T-cell marker. This construct is made with a monodonal antibody of mouse-origin, UCHT1, and a binding site mutant of DT, CRM9 (8). The immunotoxin, UCHT1-CRM9, is capable of regressing established xenografted human T-cell (Jurkat) tumors in nude mice (9). A rhesus monkey analog of UCHT1-CRM9, FN18-CRM9, was capable of not only depleting circulating T-cells but also depleting resident T-cells in the lymph nodes. This immunotoxin also delayed skin allograft rejection as compared to antibody treatment and non-treatment controls. FN18-CRM9 has also been used as an adjunct in inducing tolerance to mismatched kidney transplants (24).

In contrast with ricin and Pseudomonas exotoxin based immunotoxins, there is a potential problem using UCHT1-CRM9, or other DT-based immunotoxins, in the treatment of human diseases. Most people have been immunized against DT. Therefore these people have a pre-existing anti-DT antibody titer which could potentially inhibit or alter the efficacy of these immunotoxins. This limitation also occurred in our rhesus monkey studies. FN18-CRM9 could deplete T cells in the blood, but to a much lesser extent in animals with anti-DT antibodies, and the T cells repopulated several days earlier compared to those monkeys without anti-DT titers. In order to overcome this antibody mediated inhibition, we undertook the first examination of the effect and the mechanism of human sera containing anti-DT antibodies on UCHT1-CRM9 toxicity. A DT point mutant, a truncation mutant, and DT subfragments were used in an attempt to neutralize the anti-DT effect in human sera. Based on the neutralization data, a single-chain immunotoxin was constructed with a C-terminal deletion mutant of DT which could potentially bypass the inhibitory effect of the pre-existing anti-DT antibodies.

MATERIALS AND METHODS

Cells—Jurkat cells (ATCC) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 25 μg/ml gentamicin sulfate, and 50 μg/ml gentamicin sulfate.

Serum and Adsorbing Molecules—Goat anti-DT serum was provided by Dr. Randall K. Holmes (Uniformed Services University of Health Sciences, Bethesda, MD). Human serum samples were provided by Dr. Henry McFarland (NINDS, NIH, Bethesda, MD). CRM197, an A-subfragment mutant (Gly-Ser)38 to Glu) of DT (see Fig. 1A), with no enzymatic activity (10) was supplied by Dr. Reno Rappuoli (Biocine-IRIS, Siena, Italy). MSpA5, a truncation mutant (amino acid 385) of DT with an additional 5 amino acids at the C terminus was provided by Dr. Richard Youle (NINDS, NIH, Bethesda, MD). Purification of the DT B-subfragment has been described (11).

Immunotoxins—UCHT1-CRM9 synthesis has been described (12). The recombinant immunotoxin, sFv-DT390, was generated in two phases. First the coding sequences for the variable light (VL) and variable heavy (VH) chain regions of the UCHT1 antibody were amplified by a two-step protocol of reverse transcriptase-polymerase chain reaction using primers based on the published sequence (13). The 5′ VH primer added a unique NcoI restriction site. The 5′ VH primer added a unique NcoI restriction site. The 3′ VH primer added a termination codon at the Jd constant region junction and an EcoRI site. The Vh region was joined to the Vh region by single-stranded overlap extension and the two regions are separated by a (GlySer)4 linker that should allow for proper folding of the individual variable domains to form a function antibody binding site (14). Second, genomic DNA was isolated from a strain of Corynebacterium diphtheriae producing the DT mutant CRM9 (C7→H201Pro→203Ser→Glu65) as described (15). This DNA was used for polymerase chain reaction. The 5′ primer was specific for the toxin gene beginning at the signal sequence and added a unique Nhel restriction site. The 3′ primer was specific for the DT sequence terminating at amino acid 390 and added an Ncol site in-frame with the coding sequence. The polymerase chain reaction products were digested with the appropriate restriction enzymes and cloned into the Escherichia coli expression plasmid pET-17b (Novagen, Madison, WI). The resulting plasmids were used to transform E. coli and the cells were grown in the presence of 2 mM isopropyl β-D-thiogalactopyranoside. Proteins were isolated from the bacterial culture supernatant by gel filtration and the immunotoxin was purified by affinity chromatography using a DT affinity matrix.

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1 The abbreviations used are: DT, diphtheria toxin; ELISA, enzyme-linked immunosorbent assay; MES, 4-morpholineethanesulfonic acid.
2 D. M. Neville, Jr., J. Scharff, K. Rigaut, H. Hu, J. Shiloach, W. Slingerland, and M. Janker, submitted for publication.
incubated with Jurkat cells (5 × 10^5) that had been preincubated with serum or
for 30 min with the indicated serum sample or
antibody titers.

**ELISA** was performed in triplicate for each sample as described under "Materials and Methods." The OD values were derived from 1:100 dilutions and presented as a mean value ± SD. The background value was 0.060 ± 0.02. Titters are recorded as the highest serum dilution that showed a positive reaction in ELISA.

**UCHT1-CRM9** (2 × 10^{-18}) was incubated with different dilutions of serum for 30 min. The mixture was then added to cells as described under "Materials and Methods." Four replicates were performed for each sample. Data are presented as a mean value ± SD. In percentage of the control counts. UCHT1-CRM9 inhibited protein synthesis to 2.0% of controls. The anti-DT-antibody serum could be diluted to 1:10,000 and still completely inhibited the toxicity of **UCHT1-CRM9**.

ND, not done.

**Results**

**Serum with Anti-DT Antibodies Inhibits **UCHT1-CRM9** Tox-
ity—Since humans are immunized against DT, the presence of anti-DT antibodies in the serum was determined by ELISA (Table I). In a limited sample population, 80% of the serum samples had an anti-DT antibody titer of 1:100 or above. The vaccination status of the donors was not available. To determine the effect of these antibodies on **UCHT1-CRM9** toxicity, the immunotoxin was preincubated with different concentrations of serum and the toxicity of the mixture was assayed (Table I). Serum samples without a significant ELISA OD (2-fold above background) were incapable of affecting **UCHT1-CRM9** toxicity at high concentrations of serum (1:10). However, serum samples with a positive ELISA result could neutralize the cytotoxic effect at 1:10 dilution, and those with a high ELISA OD (7-11-fold above background) inhibited toxicity even at a 1:10 dilution. Similar results were seen in assays conducted with monkey serum samples (data not shown).

**Sera Do Not Inhibit Endocytosis of **UCHT1-CRM9**—The inhibi-
tory effect of serum on **UCHT1-CRM9** toxicity could be due to prevention of the immunotoxin binding to the cell surface or the endocytosis of **UCHT1-CRM9** into the cell. Endocytosis assays were conducted using **125I-UCHT1-CRM9** to determine if either of these processes were affected by anti-DT antibodies present in sera. The results indicate that the presence of serum (goat anti-DT or human) reduces as much as 80% of the immunotoxin counts binding to the cell surface (Table II)). While this is a significant reduction in binding, limiting 90% of input immunotoxin (one log less **UCHT1-CRM9**) in toxicity assays reduces protein synthesis to ≈25% of controls (data not shown, also see Fig. 2A). In contrast, the inhibitory effect of serum containing anti-DT antibodies is 100%. Therefore the effect of the anti-DT antibodies is not all at the level of inhibition of binding to the cell surface. The preincubation of **125I-UCHT1-CRM9** for 2 h on ice and subsequent washing at room temperature resulted in 18–25% of the total cell associated counts internalized (Table II). After incubation for 30 min at 37 °C, there is a doubling of internalized counts both with and without serum, indicating that the same percentage of labeled immunotoxin is endocytosed. The identical dilutions of serum were incubated with non-labeled **UCHT1-CRM9** and used in protein synthesis inhibition assays. The results demonstrate that the

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**Human serum with anti-DT antibodies inhibits the toxicity of **UCHT1-CRM9** and the inhibition correlates with the anti-DT titer**

| Sample | OD (X ± S.D.) | Titer | 1:10 | 1:100 | 1:1,000 | % control |
|--------|--------------|-------|------|-------|---------|----------|
| 10010  | 0.738 ± 0.017| 1:750 | 97 ± 3| 79 ± 8| 2 ± 0 |
| 10011  | 0.568 ± 0.048| 1:500 | 104 ± 2| 13 ± 2| 2 ± 0 |
| 10012  | 0.491 ± 0.025| ND a | 96 ± 3| 19 ± 2| 2 ± 0 |
| 10013  | 0.411 ± 0.052| 1:500 | 105 ± 8| 7 ± 1| 2 ± 0 |
| 10014  | 0.390 ± 0.047| 1:500 | 96 ± 2| 7 ± 0| 2 ± 0 |
| 10015  | 0.353 ± 0.008| 1:250 | 125 ± 6| 6 ± 4| 2 ± 0 |
| 10019  | 0.395 ± 0.019| 1:250 | 101 ± 7| 6 ± 1| 2 ± 0 |
| 10016  | 0.141 ± 0.015| 1:100 | 22 ± 1| 3 ± 0| 2 ± 0 |
| 10017  | 0.100 ± 0.006| <1:100| 4 ± 0| 3 ± 0| 2 ± 0 |
| 10018  | 0.071 ± 0.001| <1:100| 2 ± 0| 2 ± 0| 2 ± 0 |
| Goat   | 1.450 ± 0.013| 1:10^10| 102 ± 19| 104 ± 3|        |

a: ELISA was performed in triplicate for each sample as described under "Materials and Methods." The OD values were derived from 1:100 dilutions and presented as a mean value ± SD. The background value was 0.060 ± 0.02. Titters are recorded as the highest serum dilution that showed a positive reaction in ELISA.

b: **UCHT1-CRM9** (2 × 10^{-18}) was incubated with different dilutions of serum for 30 min. The mixture was then added to cells as described under "Materials and Methods." Four replicates were performed for each sample. Data are presented as a mean value ± SD. In percentage of the control counts. **UCHT1-CRM9** inhibited protein synthesis to 2.0% of controls. The anti-DT-antibody serum could be diluted to 1:10,000 and still completely inhibited the toxicity of **UCHT1-CRM9**.

c: ND, not done.
**sFv-DT390 Avoids Pre-existing Human Anti-DT Antibodies**

The epitopes involved in human serum's inhibition of toxicity lie in the last 150 amino acids of DT. A schematic diagram of the DT mutants CRM9, CRM197, and MSP\(\Delta S\) is presented (A). The A- and B-subfragments and their relative size and position are shown. The filled circle represents a point mutation as described in the text. Goat (B) or human (C) serum (human serum was a pool from all samples with positive ELISA for anti-DT antibodies) was incubated with increasing molar concentrations of CRM197 (\(\times\)), MSP\(\Delta S\) (\(\times\)), or the B-subfragment (\(\Delta\)) of DT for 30 min at room temperature. This mixture was then incubated with Jurkat cells (1.5 \(\times\) 10⁵) for 2 h on ice (final concentration of \(1.25\times10^{-7}\) UCHT1-CRM9 was 1 \(\times\) 10⁻¹⁰). The cells were then washed and endocytosis assays were performed as described under "Materials and Methods." The % Bound value represents the cell associated counts divided by the cell associated counts without serum. Non-labeled UCHT1-CRM9 was incubated with the above dilutions of sera and the resulting mixture was used in protein synthesis inhibition assays. The results shown are representative of two independent assays.

| Serum sample | Time (37°C) | % Bound | % of bound internalized | Protein synthesis |
|--------------|-------------|---------|------------------------|------------------|
|               | min         |         |                        |                  |
| Human         | 0           | 100     | 23.6                   | ND \(^a\)        |
| Human         | 30          | 100     | 58.8                   | 3 ± 1           |
| Goat          | 0           | 37      | 24.4                   | ND               |
| Goat          | 30          | 33      | 50.7                   | 92 ± 14         |

\(^a\) ND, not done.

**Fig. 1.** The epitopes involved in human serum's inhibition of toxicity lie in the last 150 amino acids of DT. A schematic diagram of the DT mutants CRM9, CRM197, and MSP\(\Delta S\) is presented (A). The A- and B-subfragments and their relative size and position are shown. The filled circle represents a point mutation as described in the text. Goat (B) or human (C) serum (human serum was a pool from all samples with positive ELISA for anti-DT antibodies) was incubated with increasing molar concentrations of CRM197 (\(\times\)), MSP\(\Delta S\) (\(\times\)), or the B-subfragment (\(\Delta\)) of DT for 30 min at room temperature. This mixture was then incubated with Jurkat cells (1.5 \(\times\) 10⁵) for 2 h on ice (final concentration of \(1.25\times10^{-7}\) UCHT1-CRM9 was 1 \(\times\) 10⁻¹⁰). The cells were then washed and endocytosis assays were performed as described under "Materials and Methods." The % Bound value represents the cell associated counts divided by the cell associated counts without serum. Non-labeled UCHT1-CRM9 was incubated with the above dilutions of sera and the resulting mixture was used in protein synthesis inhibition assays. The results shown are representative of two independent assays.

**TABLE II**

Inhibition of UCHT1-CRM9 toxicity by serum does not correlate with inhibition of endocytosis

\(1\times10^{-4}\) UCHT1-CRM9 (2 \(\times\) 10⁻⁷ \(M\)) was incubated with medium or anti-DT serum (1:4 dilution of human sample 10010 or a 1:1,000 dilution of goat serum; Table I) for 30 min at room temperature. This mixture was then incubated with Jurkat cells (1.5 \(\times\) 10⁵) for 2 h on ice (final concentration of \(1.25\times10^{-7}\) UCHT1-CRM9 was 1 \(\times\) 10⁻¹⁰). The cells were then washed and endocytosis assays were performed as described under "Materials and Methods." The % Bound value represents the cell associated counts divided by the cell associated counts without serum. Non-labeled UCHT1-CRM9 was incubated with the above dilutions of sera and the resulting mixture was used in protein synthesis inhibition assays. The results shown are representative of two independent assays.

| Serum sample | Time (37°C) | % Bound | % of bound internalized | Protein synthesis |
|--------------|-------------|---------|------------------------|------------------|
|               | min         |         |                        |                  |
| Human         | 0           | 100     | 23.6                   | ND \(^a\)        |
| Human         | 30          | 100     | 58.8                   | 3 ± 1           |
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| Goat          | 30          | 33      | 50.7                   | 92 ± 14         |

\(^a\) ND, not done.
sFv-DT390 Avoids Pre-existing Human Anti-DT Antibodies

DISCUSSION

The current investigation is the first analysis on the effect of pre-existing anti-DT antibodies on DT-based immunotoxins and to determine the mechanism of the observed effect. Our results indicate that the pre-existing anti-DT antibodies present in human serum inhibit the toxicity of the immunotoxin UCHT1-CRM9. This inhibition of toxicity was also observed with goat anti-DT serum, however, less goat serum was needed to completely inhibit toxicity. The experiments were designed in such a way to mimic the in vivo situation. The peak concentration of circulating immunotoxin currently being tested in animal models is $1 \times 10^{-9}$ M, thus approximating in vivo conditions. The inhibition of toxicity correlates with the serum antibody levels as determined by ELISA (Table I), indicating that sera with higher anti-DT titers have a stronger inhibitory effect. Similarly, the goat anti-DT serum which gave the highest ELISA value could be diluted 10,000 times and still completely inhibited UCHT1-CRM9 toxicity. Since this correlation exists, there is no indication that any other component of the serum inhibits the toxicity of UCHT1-CRM9. Furthermore, our data show that a titer of 1:100 dilution is necessary for an inhibition of the immunotoxin toxicity. This is in agreement with data from a clinical trial (20). A construct in which the first 486 amino acids of DT were fused to interleukin-2, DAB$_{486}$IL-2, was used in lymphoid malignancy patients. A partial response to DAB$_{486}$IL-2 was observed in several patients who had a anti-DT titer below 1:100 dilution prior to the treatment.

Intoxication of cells by immunotoxins can be subdivided into four general stages: 1) specific binding to the cell surface, 2) endocytosis into the cell, 3) translocation of enzymatic domain of the toxin out of the endosome, and 4) enzymatic inactivation of the target molecule. The results presented indicate that, while the amount of immunotoxin reaching the cell surface is lower in the presence of serum, the same percentage of bound immunotoxin is endocytosed. Taking into account the reduced amount of immunotoxin bound to the cell, the amount of endocytosed immunotoxin should intoxicate the cells to below 25% of controls. However, the immunotoxin had no effect on protein synthesis in the presence of serum containing anti-DT antibodies. Since the A-subfragment of DT could not adsorb the protective effect of serum while the B-subfragment could, the effect of serum is not likely to be at the level of inhibiting enzymatic activity of the toxin. Therefore, it suggests that the anti-DT antibodies affect the translocation of the A-subfragment into the cytosol.

CRM197, B-subfragment, and MSP$_{35}$ could adsorb the protecting anti-DT antibodies from the goat and rhesus monkey (data not shown) sera. However, among the 3 DT mutants, MSP$_{35}$ could not prevent the UCHT1-CRM9 toxicity in the presence of the human sera, showing a difference in the anti-DT antibody repertoire among humans, goat, and rhesus monkeys. This difference does not seem to be due to immunization routes, because monkeys used in the present study were not immunized for DT and presumably acquire the antibodies after a natural infection with toxigenic strains of C. diphtheriae. Although there were reports showing that rhesus monkeys and humans shared a similar antibody repertoire (21), our results suggest that one must analyze the effect of antibodies from the host for whom immunotoxin treatment is intended.

To overcome the blocking effect of the pre-existing anti-DT antibodies in human sera, there are basically two pathways existing. One is to neutralize the antibodies with non-toxic DT mutants, and the other is to modify the DT structure used for making immunotoxin (3). The antibody neutralization pathway has been tested in our monkey studies of FN18-CRM9 treatment. A 100-fold higher amount of CRM197 was injected 5 min before FN18-CRM9 to adsorb the pre-existing antibodies in 2 monkeys who had an anti-DT titer at 11,000 dilution. In one monkey the T cell depletion was as good as in monkeys without anti-DT titers. The other monkey died due to multiple kidney infarcts. It is possible that this condition resulted from immune complex disease precipitated by the neutralization procedure. Thus, serum neutralization may be a potentially dangerous process. Our results showed that although antibodies against

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both A- and B-subfragments existed in human sera, MSP35 could not neutralize the pre-existing protective anti-DT antibodies, and therefore could not prevent the inhibition of the cytotoxicity of UCHT1-CRM9. However, it did block the inhibitory effect of the goat and monkey sera. This prompted the construction of the recombinant immunotoxin, sFv-DT390. The IC\textsubscript{50} of sFv-DT390 is 4.8 \times 10^{-11} M, 1/16 as potent as UCHT1-CRM9. Like many other single-chain constructs, sFv-DT390 is monovalent as compared to immunotoxins generated with full-length bivalent antibodies. The reduced toxicity in sFv-DT390 could be explained primarily on this affinity difference. Immunotoxins generated with purified Fab(\textsubscript{ab})\textsubscript{2} fragments of antibodies also show an in vitro loss in toxicity (generally a 1.5 log difference) when compared to their counterparts generated with full-length antibodies (22). The toxicity of sFv-DT390 is comparable to that reported for DAB\textsubscript{SGD}-L2 (23). Considering using sFv-DT390 in the clinical treatment, will there be a trade-off in evaluating a potent, but completely neutralized toxin with one which is less potent, but not completely blocked? A conclusive answer can only be derived from clinical trials. However, from the in vitro data some advantages of sFv-DT390 can be expected. First, sFv-DT390 is only one-third of the molecular weight of UCHT1-CRM9. Therefore, sFv-DT390 can penetrate into tissue more readily. Second, in an in vitro experiment (Table III), the same molar concentration of sFv-DT390 and UCHT1-CRM9 was used for the serum inhibition test, although the former is only 1/16 potenter compared to the latter. The pre-existing anti-DT antibodies in human sera could only partially block the toxicity of sFv-DT390 while the effect of UCHT1-CRM9 was completely blocked. Thus, sFv-DT390 could potentially bypass the anti-DT antibodies in vivo situations while UCHT1-CRM9 cannot. Third, sFv-DT390 contains only the variable region of UCHT1 and should have less immunogenicity in human anti-mouse antibody responses than the native murine antibody UCHT1. Finally, the production cost of sFv-DT390 is much lower than that of UCHT1-CRM9. Based on these reasons, it is conceivable that sFv-DT390, or others with similar properties, should be useful in the treatment of T-cell mediated diseases in humans, especially in anti-DT positive individuals and in patients who need repeated treatments. To obtain evidence supporting this assumption, a rhesus monkey analog of sFv-DT390 is currently being constructed in this laboratory, and will be tested in monkey models.

In summary, this report demonstrates that human sera can affect the toxicity and therefore the efficacy of immunotoxins generated with full-length DT mutants. It also indicates that there is a difference in anti-DT antibody repertoire between the humans and non-human primates, suggesting the immunotoxins destined for clinical trials be investigated with the appropriate serum. Furthermore, a truncated DT mutant has been suggested for generation of immunotoxins to bypass the blocking effect of pre-existing anti-DT antibodies in humans.

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