Effect of pre-existing anti-diphtheria toxin antibodies on T cell depletion levels following diphtheria toxin-based recombinant anti-monkey CD3 immunotoxin treatment

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1. Introduction

Anti-CD3 immunotoxins (CD3-IT) are valuable in their ability to specifically deplete T-cells without disturbing other immune cell types. CD3-ITs are often constructed using diphtheria toxin (DT), a 535 amino acid exotoxin, capable of crossing eukaryotic cell membranes and initiating cell death via inhibition of protein synthesis. Full-length DT is made up of three functionally different domains: a catalytic domain responsible for the inhibition of protein synthesis, a transmembrane domain whose conformational change allows for entry into the endosomal membrane permitting the catalytic domain to enter the cytoplasm, and a receptor binding domain which binds the cell surface receptor [1,2]. Recently, construction of immunotoxins using a truncated diphtheria toxin lacking the receptor binding domain has been most optimal. The single-chain construct offers several advantages over its full-length counterpart including a lower molecular weight allowing for easier access into tissue, as well as reduced toxicity [3].

In preclinical and clinical studies, anti-CD3 immunotoxins have been used for the treatment of T-cell lymphomas in humans [4] and as an agent for T-cell depletion in experimental transplantation studies in animal models [5–7]. Pre-existing antibodies to DT as a result of vaccination or infections with *C. diphtheriae* may interfere or inhibit the function of these anti-CD3 immunotoxins. Previously, a full-length anti-rhesus monkey CD3 immunotoxin, FN18-CRM9, was shown to be less effective at depleting circulating T cells in animals with pre-existing anti-DT antibody titers than in animals without antibodies, and subsequent doses were ineffective. In this study, the T cell depletion function of a truncated DT based recombinant anti-monkey CD3 immunotoxin, A-dmDT390-scfbDb (C207), as part of a reduced intensity conditioning regimen prior to hematopoietic cell transplantation, was compared between two groups of monkeys: those with and without pre-existing anti-diphtheria antibodies. T cell depletion was comparable in both groups of monkeys, and therefore appeared to be unaffected by the presence of moderate levels of pre-existing anti-diphtheria antibodies.

Diphtheria toxin (DT)-based anti-CD3 immunotoxins have clinical relevance in numerous applications including autoimmune disease therapies and organ transplantation tolerance protocols. Pre-existing anti-DT antibodies acquired either by vaccination against diphtheria toxin or infections with *C. diphtheriae* may interfere or inhibit the function of these anti-CD3 immunotoxins. Previously, a full-length anti-rhesus monkey CD3 immunotoxin, FN18-CRM9, was shown to be less effective at depleting circulating T cells in animals with pre-existing anti-DT antibody titers than in animals without antibodies, and subsequent doses were ineffective. In this study, the T cell depletion function of a truncated DT based recombinant anti-monkey CD3 immunotoxin, A-dmDT390-scfbDb (C207), as part of a reduced intensity conditioning regimen prior to hematopoietic cell transplantation, was compared between two groups of monkeys: those with and without pre-existing anti-diphtheria antibodies. T cell depletion was comparable in both groups of monkeys, and therefore appeared to be unaffected by the presence of moderate levels of pre-existing anti-diphtheria antibodies.

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2. Materials and methods

2.1. Conditioning regimen and HCT

Prior to hematopoietic cell transplantation on day 0, recipient monkeys were treated with 8 doses (25 μg/kg/dose) of an anti-monkey CD3 recombinant immunotoxin [10] administered twice daily over 4 days prior to transplantation (day −4 to −1), low dose (100 cGy) whole body irradiation (day −2), and a 45 day course of Cyclosporine A (day 0 to 45) (Pathiraja et al. manuscript in preparation).

2.2. Anti-DT ELISA

We measured pre-existing and induced anti-CD3 immunotoxin IgG by ELISA adapted from Woo et al. [11]. ELISA Maxisorp plates (Nunc, Rochester, NY, USA) were coated with 100 μL/well of the anti-monkey CD3-immunotoxin at 5 μg/ml in PBS. Plates were washed with PBS (Cellgro, Manassas, VA) + 0.1% Tween 20 (polyoxyethylene)sorbitan monolaurate Sigma–Aldrich, St. Louis, MO, USA) and blocked 3% Gelatin (Type B: From Bovine Skin, Sigma) in PBS. Serial dilutions (1:10; 1:100; 1:1000; 1:10,000) of rhesus macaque serum in triplicates (10 μL) were loaded in the blocked plates and incubated at 37 °C for 1 h. Goat anti-DT (Serotec) was used to generate a standard curve for measuring levels of anti-DT in sera. The plates were probed with rabbit anti-human IgG HRP or rabbit anti-goat HRP for 1 h at 37 °C, developed with ABTS (Southern Biotech, Birmingham, Al), and read at 405 nm. Results are presented as OD values and antibody titers. Serum samples measured following immunotoxin administration were measured at a single time point between 19 and 125 days following the final dose.

2.3. Immunofluorescence staining and flow cytometric analysis

Flow cytometry (FACS) analysis was performed using a Becton Dickinson FACScan (San Jose, California, USA). Heparinized peripheral blood was distributed into staining tubes (Falcon 2054) at 100 μL/tube and washed once using 2 mL flow cytometry buffer (HBSS containing Ca2+ and Mg2+/0.1% BSA). Cells were then stained with 10 μL of direct fluorescein isothiocyanate (FITC)-labeled CD3 (SP34) or phycoerythrin-streptavidin (PE)-conjugated CD3 (SP34) mAb for 30 min at room temperature. RBCs were lysed and the cells were fixed using FACS lysing solution (Becton Dickinson) before acquisition. Data were analyzed using Winlist mode analysis software (Verity Software House, Topsham, Maine, USA).

3. Results

3.1. T cell depletion was comparable in monkeys with and without detectable levels of pre-existing anti-DT IgG titers

Of the eight monkeys analyzed in this study (Table 1), three (Group 1) had anti-DT serum antibody levels not significantly different than background levels at 1:100 dilution. Five monkeys (Group 2) had significant (2-fold above background) levels of anti-DT antibody prior to treatment with the recombinant anti-monkey CD3 immunotoxin administration without prior immunization record against DT. CD3+ T cell levels were measured by flow cytometry immediately prior to the first dose of the recombinant anti-monkey CD3 immunotoxin, and after four doses and eight doses (Fig. 1). The average numbers of CD3+ T-cells prior to conditioning were similar between the two groups of monkeys (group 1 = 2677 cells/μL vs. group 2 = 2988 cells/μL). Following four doses of the recombinant anti-monkey CD3 immunotoxin, the CD3+ T cell levels declined in both groups to an average of 178 cells/μL in group 1 and 222 cells/μL in group 2. CD3+ T cell levels in both groups following eight doses of immunotoxin and 100 cGy TBI were below 100 cells/μL (28 and 44).

3.2. Anti-DT antibody develops following recombinant anti-monkey CD3 immunotoxin administration in monkeys without pre-existing anti-DT antibody

Prior to hematopoietic cell transplantation, the recombinant anti-monkey CD3 immunotoxin was administered as part of the conditioning regimen. Subsequently, serum was analyzed by ELISA for increases in anti-DT Ab titers (Fig. 2). All three of the animals without significant levels of anti-DT antibody prior to immunotoxin administration displayed an increase in the OD values read at 1:100 serum dilution and in the highest serum dilution that showed a positive reaction. Serum from monkeys with pre-existing anti-DT antibody titers did not show an overall increase in OD values nor the highest serum dilution that showed a positive reaction.

4. Discussion

Anti-CD3 immunotoxins have clinical relevance for numerous applications including autoimmune disease therapies, T-cell leukemias, and organ transplantation. We have developed a novel reduced intensity conditioning regimen which involves the use of the recombinant anti-monkey CD3 immunotoxin for T cell depletion for the induction of transplantation tolerance (Pathiraja et al. manuscript in preparation). Whereas many humans develop anti-DT titers as a result of direct immunizations against DT, some rhesus macaques may acquire anti-DT titers as a result of infections with toxigenic strains of C. diphtheriae [3] or other cross-reactive antibodies. Pre-existing circulating anti-DT antibodies could potentially have a neutralizing effect on DT-based immunotoxins. In this brief report, we provide evidence for exceptional T cell depletion using a recombinant anti-monkey CD3 immunotoxin despite the presence of moderate levels of pre-existing anti-DT antibodies found in the naïve rhesus macaques available to study.

Previously, the use of a full-length DT based immunotoxin in rhesus macaques with pre-existing Ab was shown to be less effective in depleting T-cells in the blood and delaying T cell repopulation [8]. In a study by Neville et al., the full-length anti-rhesus monkey CD3 immunotoxin, FN18-CRM9, constructed by chemically conjugating a ---

Table 1

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Pre OD</th>
<th>Titer</th>
<th>Concentration (ng/mL)</th>
<th>Post OD</th>
<th>Titer</th>
<th>Concentration (ng/mL)</th>
<th>Pre anti-CD3 IT (cells/μL)</th>
<th>Day 2 (cells/μL)</th>
<th>Day 4 (cells/μL)</th>
</tr>
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<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>S0090</td>
<td>0.269 ± 0.047</td>
<td>&lt;1:10</td>
<td>&lt;100</td>
<td>1.758 ± 0.220</td>
<td>1:1000</td>
<td>238,720</td>
<td>2,480</td>
<td>269</td>
<td>26</td>
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<tr>
<td>S0409</td>
<td>0.193 ± 0.019</td>
<td>&lt;1:10</td>
<td>&lt;100</td>
<td>0.502 ± 0.040</td>
<td>1:1000</td>
<td>2,277</td>
<td>3,181</td>
<td>88</td>
<td>18</td>
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<tr>
<td>S0609</td>
<td>0.262 ± 0.079</td>
<td>1:100</td>
<td>1:100</td>
<td>0.414 ± 0.066</td>
<td>1:1000</td>
<td>1,467</td>
<td>2,371</td>
<td>N/A</td>
<td>41</td>
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<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S0610</td>
<td>0.348 ± 0.224</td>
<td>1:100</td>
<td>1,842</td>
<td>0.363 ± 0.001</td>
<td>1:1000</td>
<td>1,178</td>
<td>2,146</td>
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<tr>
<td>S1010</td>
<td>0.571 ± 0.093</td>
<td>1:100</td>
<td>2,354</td>
<td>0.713 ± 0.024</td>
<td>1:1000</td>
<td>2,391</td>
<td>3,406</td>
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<td>26</td>
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<tr>
<td>SB110</td>
<td>1.646 ± 0.116</td>
<td>1:1000</td>
<td>6,423</td>
<td>1.378 ± 0.061</td>
<td>1:1000</td>
<td>6,588</td>
<td>2,455</td>
<td>180</td>
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<tr>
<td>S9110</td>
<td>0.641 ± 0.143</td>
<td>1:1000</td>
<td>1,300</td>
<td>0.389 ± 0.020</td>
<td>1:1000</td>
<td>1,195</td>
<td>3,026</td>
<td>255</td>
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</tr>
<tr>
<td>S8610</td>
<td>0.719 ± 0.003</td>
<td>1:100</td>
<td>697</td>
<td>0.645 ± 0.005</td>
<td>1:1000</td>
<td>2,106</td>
<td>3,908</td>
<td>125</td>
<td>16</td>
</tr>
</tbody>
</table>
binding site mutant of diphtheria toxin, CRM9 and an anti-rhesus monkey CD3 mAb, FN18, depleted T cells to a "much lesser extent" in monkeys with detectable anti-CRM9 titers at a 1:100 dilution of sera [8]. Furthermore, subsequent doses were without effect and T-cell repopulation was observed earlier in those monkeys.

As part of this study, we assessed the effect of pre-existing anti-DT antibodies on the T cell depletion function of the recombinant anti-monkey CD3 immunotoxin, A-dmDT390 scFvDb (C207), which consists of "the catalytic and translocation domains of DT (DT390)" fused to the affinity matured "single-chain fold-back diabody of FN18" [9]. Studies in humans have shown that compared to a full-length anti-human CD3-immunotoxin, UCHT1-CRM9, which was completely neutralized by anti-DT antibodies in human sera detectable at 1:100 dilutions, a recombinant anti-human CD3 immunotoxin A-dmDT390scFv (UCHT1) was only moderately inhibited under the same conditions [3]. In a recent clinical trial assessing the therapeutic effects of anti-CD3 recombinant diphtheria immunotoxin treatment on cutaneous T cell lymphoma in five patients, there was no association of pre-treatment antibody titer with Cmax, the peak immunotoxin serum level, after infusion [12]. All patients in the study had received immunizations against DT during childhood, resulting in a positive signal by enzyme immunoassay. Concentrations of circulating anti-DT antibodies prior to receiving any immunotoxin were calculated between 1 and 5 μg/mL, similar to those observed in this study. Concentrations of anti-DT antibodies increased following immunotoxin treatment in all patients.

Our data demonstrate that T cell depletion function of the recombinant anti-monkey CD3 immunotoxin is unaffected by the presence of moderate levels of pre-existing anti-DT antibodies in naïve rhesus macaques. Peripheral blood CD3+ levels in both groups of monkeys following 4 and 8 doses (100 μg/kg total) of anti-monkey CD3 immunotoxin were depleted equally. It is possible that the presence of higher levels of anti-DT antibody may interfere with depletion function; however, our study could not address this due to lack of availability of animals with higher titers. These data may help facilitate the use of the recombinant anti-monkey CD3 immunotoxin in rhesus macaque models of transplantation.

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**References**