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Recombinant anti-monkey CD3 immunotoxin depletes peripheral lymph node T lymphocytes more effectively than rabbit anti-thymocyte globulin in naïve baboons

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Abstract

T cell depletion is an important procedure for both experimental and therapeutic immune modulation. Rabbit anti-thymocyte globulin (ATG), which is a commonly used T cell depletion antibody in clinical organ and cell transplantation protocols, is effective in temporarily depleting peripheral blood T lymphocytes but only moderately effective in depleting peripheral lymph node T cells which comprise the majority of T lymphocytes. A recombinant anti-CD3 immunotoxin, A-dmDT390-scfbDb (C207), has been developed and shown in an initial study to retain the lymph node depleting properties of conjugated CD3 immunotoxin. This agent could potentially be used synergistically with or as a replacement for rabbit ATG in preclinical primate models of transplantation. We directly compared the peripheral blood and lymph node depleting abilities of this recombinant anti-CD3 immunotoxin and rabbit ATG in naïve animals at clinically tolerated doses. Baboons were treated with a full course of either rabbit ATG (n = 2) or CD3 immunotoxin (n = 3). Peripheral blood and lymph node T lymphocytes were measured before and following treatment. Peripheral blood CD3+ cells fell below 100 cells/ μ L in every animal. In the two animals receiving ATG, CD3+ cells represented 53% and 68% of lymph node cells two days following a full course of rabbit ATG. In contrast, CD3+ cells represented 3%, 5%, and 38% in lymph nodes following a full course of CD3-IT. Thus, recombinant anti-monkey CD3 immunotoxin showed improved peripheral lymph node T lymphocyte depletion to rabbit ATG and spared other immune cells.

Keywords

T-cell; ATG; Immunotoxin; Depletion; Lymph node

1. Introduction

For the last thirty years, monoclonal and polyclonal antibody therapies that either deplete alloreactive T lymphocytes or impede their activation have been used successfully to prevent or treat allograft rejection [1,2]. In clinical organ transplantation, antibody treatment

has led to significantly improved graft survival among renal and liver transplant patients. It has allowed for delayed use or lower doses of global immunosuppressants like azathioprine and cyclosporine. In bone marrow transplantation, T cell depletion has provided clinicians with a powerful tool for the management of GVHD [3,4]. Further, anti-T cell antibody treatment has played a key role in the successful induction of transplant tolerance both in animal studies and in human clinical trials using mixed chimerism [5].

Rabbit ATG, perhaps the most established anti-T cell agent currently in use, is effective in temporary depletion of circulating peripheral, as well as graft infiltrating T lymphocytes [6]. However, it is less successful in peripheral lymph node T cell depletion in primates. It has been shown to bind with less avidity to lymphocytes within the lymph node, and this may be a contributing factor [7].

Peripheral lymph nodes play a pivotal role in T cell trafficking and are the site where antigen-presenting cells (APC) present antigen to naïve T lymphocytes. They harbor the majority of resting T cells and central memory T cells and are an important site for T cell/B cell interaction [8]. Because of this role in the mobilization of primary and secondary effector responses to foreign antigens, anti-T lymphocyte antibodies that are able to temporarily deplete in the peripheral lymph node may provide improved outcomes in the prevention of graft rejection and in the achievement of tolerance through mixed chimerism.

A chemically conjugated monkey anti-CD3 immunotoxin (FN18-CRM9) is known to deplete both peripheral blood and lymph node T cells [9,10]. Transplantation studies using this reagent in monkeys showed improved graft survival of renal grafts [11–14]. Due to low production yields of the above agent, a recombinant version with higher T cell affinity and greater bioactivity in a DT based immunotoxin compared to FN18 was developed [15]. In a fold-back diabody format, affinity matured FN18 scFv (C207) has a 7-fold increase in T cell binding and showed a profound decrease in lymph node T cells from 66% to 18.7% in one treated rhesus macaque [16].

Since that initial study, no further studies have specifically addressed this potential strength of recombinant A-dmDT390-scfbDb (C207) anti-CD3 immunotoxin based therapy in primate models of transplantation. And yet, T cell depletion during the induction period has been postulated to be a key component of tolerance induction following transplantation [5]. Two thymectomized baboons in our pig-to-baboon xenotransplantation studies showed excellent lymph node depletion using a regimen that included a full 8 dose course of A-dmDT390-scfbDb (C207) recombinant anti-CD3 immunotoxin [17]. We therefore set out to examine whether this effect would be seen in naïve animals treated with this agent alone and how this compared to ATG.

Results from this study will help to inform the design of conditioning regimen in future primate studies of transplantation tolerance. Further, even though A-dmDT390-scfbDb (C207) is species-specific to monkeys, we have observed excellent lymph node T cell depletion using the swine equivalent agent developed in our lab [18] demonstrating that this effect can be observed across species. The human equivalent agent, A-dmDT390-bisFv (UCHT1), is currently undergoing stage 1 trials for the treatment of T cell lymphomas (ClinicalTrials.gov Identifier: NCT00611208). If it is demonstrated to be safe for human use, results from primate studies using A-dmDT390-scfbDb (C207) could potentially be extrapolated into human studies.

2. Materials and methods

2.1. Animal care and treatment

Six naïve *Papio hamadryas* baboons (2–4 years old) from Mannheimer Foundation, Homestead, FL were treated. These baboons had previously received skin transplants as part of another study, but never received any immunosuppressive nor T cell depleting agents prior to this study. Additionally, we waited a period of 3–6 weeks following previous experiments and until all hematologic and immune parameters were normal before use in this study. All experiments were approved and performed in compliance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital.

Between 3 and 7 days prior to administering T cell depleting agents, indwelling central venous catheters were surgically inserted and sustained using a flexible tether/jacket system, as previously described [19]. This system allowed easy intravenous injection of T cell depleting antibodies and monitoring of peripheral T cell counts. At the time of central venous catheter insertion, a pre-treatment inguinal lymph node was harvested from each animal for the measurement of baseline T cell numbers. Preliminary studies had shown that the inguinal region provided a consistently representative lymph node distribution comparable to other regional lymph node areas.

2.2. T cell depletion

Two baboons ($n = 2$) received treatment with commercially available rabbit anti-thymocyte globulin (ATG) (Thymoglobulin, Genzyme Cambridge, MA) at 20 mg/kg on two consecutive days. Each dose was administered as a continuous intravenous infusion in normal saline over 4–6 h.

Four baboons ($n = 4$) received recombinant anti-monkey CD3 immunotoxin, A-dmDT390-scfbDb (C207), that has been previously described [16]. Three of the four baboons received 8 doses of 25 µg/kg twice daily on four consecutive days (full course immunotoxin) and one of the baboons received single bolus dose of 50 µg/kg for comparison. In either case, CD3 immunotoxin was administered as an IV push. All baboons received pre-treatment with antihistamine (Benadryl, 0.5 mg/kg) 15 min prior to drug infusion (Table 1).

2.3. T cell lymphocyte monitoring

T cell numbers were monitored quantitatively by flow cytometry and qualitatively by immunohistochemistry (IHC) prior to antibody treatment and two days after treatment completion.

2.4. Flow cytometry

Pre-treatment and post-treatment lymph nodes were placed in saline after excision and were crushed and suspended in HBSS within 2 h. They were then sieved through a 100-micron sieve. Fluorescence-activated cell sorter (FACS) analysis of CD3⁺ and CD20⁺ cells was performed using a Becton Dickinson FACScan (San Jose, California, USA) as described previously [19]. Briefly, heparinized peripheral blood or processed lymph node cells (10^7 cells/mL) were distributed into staining tubes (Falcon 2054) at 100 µL/tube and washed once using 2 mL flow cytometry buffer. Cells were then stained with 10 µL of FITC mouse anti-human CD3 (SP34 clone from BD Pharmingen and FITC mouse anti-monkey CD3 FN18 clone [20] a kind gift of Dr. David Neville) as well as PE mouse anti-human CD20 (BD Pharmingen) for 30 min at room temperature (peripheral blood) or at 4 °C (lymph node cells). Peripheral blood red cells were lysed with ACK media for 2 min and the remaining cells were washed twice. Samples were read on FACScan and data were analyzed using

WinList list mode analysis software (Verity Software House, Topsham, Maine, USA). We stained the baboon cells with two different anti-CD3 antibodies (SP34 and FN18) which detect two different epitopes of the CD3 molecule and were satisfied that they consistently gave equivalent T cell counts, thus minimizing the risk of erroneous results due to antibody binding without cell killing.

2.5. Immunohistochemistry

Lymph node samples were immediately placed in formalin and processed as routine histologic samples. Antigen retrieval (Diva decloaking solution, Biocare Medical) was performed on paraffin embedded sections before dual staining for CD3 (rabbit anti-human CD3, DAKO; biotinylated goat anti-rabbit IgG, Vector Labs; streptavidin, Biogenix; DAB, DAKO) and CD20 (mouse anti-human CD20, DAKO; biotinylated horse anti-mouse IgG, Vector Labs; AP blue, Vector Labs).

3. Results

3.1. Rabbit ATG and CD3 immunotoxin depleted T cells equally in the peripheral blood

Prior to depletion, the mean T-cell level in baboons was 2860 cells/ μ L, with a range of 1725–4532 (Fig. 1A). Following one dose of rabbit ATG, T cell levels fell below 300 and following the second dose, T cells were below 100 in both animals. T cell levels were monitored for 2 weeks in one animal (B6311) and remained below 400, demonstrating that peripheral T cell depletion was sustained (Fig. 1C). By comparison, T cell levels in animals receiving a full course of CD3 immunotoxin (200 μ g/kg over 4 days) fell below 30 in all three animals (B0411, B0511, B6111). T cell levels in recipients of CD3-IT were more transiently depleted compared to rabbit ATG, increasing to greater than 800 at 2 weeks. A single bolus dose of 50 μ g/kg of CD3 immunotoxin resulted in a T cell decrease from 2163 to 214, demonstrating that a single administration of CD3 IT was adequate to deplete T cells transiently in the peripheral blood.

3.2. B cells were unaffected by CD3 immunotoxin but not rabbit ATG

Peripheral blood B cells were generally unaffected by T cell depletion with CD3-IT (Fig. 1B). In animals treated with a full course of CD3-IT the mean B cell count was decreased from 1330 cells/ μ L to 707 cells/ μ L. In contrast, the mean B cell count in animals treated with rabbit ATG was decreased from 1252 cells/ μ L to 162 cells/ μ L. The effect on the absolute WBC count was also markedly different (Fig. 1D). The WBC in animals receiving rabbit ATG fell below 1300 cells/ μ L, while the WBC in animals receiving CD3 IT never fell below 6000 cells/ μ L.

3.3. CD3 immunotoxin was superior to rabbit ATG in peripheral lymph node depletion

Although T cell depletion in the peripheral blood was comparable between the two agents, there was a marked difference between the two agents in lymph node depletion (Fig. 2A). CD3⁺ cells were the predominant cells in lymph nodes prior to depletion ranging from 61.6% to 85.5% of all leukocytes. In the two animals receiving ATG, the percentage of CD3⁺ cells in lymph nodes decreased to 53% and 68% following a full course of treatment. In contrast, CD3⁺ cells represented 3%, 5%, and 38% of leukocytes in lymph nodes following a full course of CD3-IT. Additionally, histologic examination demonstrates near complete depletion of CD3 cells from a peripheral lymph node after a full course of CD3 immunotoxin (Fig. 2B). In addition, there was markedly decreased paracortical cellularity by H&E stain (not shown), suggesting true depletion rather than a blockade in antibody binding. Animals treated with ATG had minimal to no qualitative depletion by CD3

immunohistochemistry or H&E stains. A single bolus dose of 50 µg/kg of CD3-IT was insufficient to deplete T cells in the lymph node (not shown).

4. Discussion

The temporary depletion of lymphocytes is of established use and benefit in clinical transplantation [1,2]. Anti-thymocyte globulins have perhaps been the most successful, while the use of monoclonal antibodies like OKT3, campath-1H, and alefacept has been limited by concerns about clinical safety [21–23].

While the immunosuppressive and peripheral T cell depleting effects of rabbit ATG are well known, it has been found to be less effective at lymph node T cell depletion [7]. Equine ATG likewise showed limited ability for peripheral lymph node depletion although strategies to further limit T cell responses have allowed tolerance induction to transplanted renal grafts [24–26].

In its original chemically conjugated format, anti-monkey CD3 (FN18) immunotoxin depleted both peripheral and lymph node T cells plus facilitated prolonged graft survival when used in transplant models [9,13,14]. A recombinant version with better production yields and bioactivity was produced [15] and in one rhesus macaque demonstrated to have LN depleting function [16].

In this study we reinforce those findings by showing that three baboons treated with recombinant CD3 immunotoxin clearly and specifically deplete lymph node T cells while sparing other cells in 3 baboons treated with a full eight dose course of monkey anti CD3 immunotoxin. We also demonstrate that as compared to rabbit ATG, anti-monkey CD3 immunotoxin depletes peripheral lymph nodes more effectively while showing comparable peripheral blood depletion. Both agents had comparable rates of T cell recovery in two weeks of follow-up. This is the first time as far as we know that the effects of the two agents on peripheral blood T cells, lymph node T cells and on T cell recovery have been tested and it provides valuable information for designing of future primate transplantation studies. Findings may also be extrapolated to human studies since the human equivalent is a very similar agent with the only difference being that human specific bivalent scFv (UCHT1) is utilized for the binding portion instead of monkey specific C207.

Clearly, near complete depletion of all T cells during the induction phase is on its own unlikely to allow long-term graft acceptance in transplantation models. And the effects with redosing of anti-CD3 immunotoxin have yet to be tested. However this transient near complete depletion even in the lymph nodes may provide a powerful tool in tolerance induction protocols either as a replacement for rabbit ATG or in adjunct.

At two days after completion of a full dose treatment, when we have usually observed the most profound peripheral blood depletion, we demonstrate similarly good lymph node depletion using anti-CD3 immunotoxin. Further, it is during this period that the initial host–graft interactions like APC antigen sampling, migration to nodes, and presentation to T cells, are known to take place. Tolerance studies comparing rabbit ATG and immunotoxin as T cell depletion agents in induction protocols will provide useful answers as regards the role of lymph node T cells in tolerance induction.

A limitation of our study was that we only focused on the global T cell numbers even though it is clear that the immunosuppressive and immunomodulatory effects of anti-lymphocyte targeted agents are not limited strictly to the global depletion of T cells. Further, it remains to be seen how well T cells are depleted in other lymphoid organs, including the thymus and spleen by anti CD3 immunotoxin. These issues need to be addressed in future studies. The

value of this work is in demonstrating the superior ability of a recombinant anti-monkey CD3 recombinant immunotoxin to deplete peripheral LN T cells in naïve baboons as a lone treatment while retaining comparable peripheral depletion ability and similar T cell return kinetics. This difference, in comparison to rabbit ATG, is achievable at clinically relevant and well-tolerated doses, without the added effects of other treatments and is supported both by flow cytometric assessment and immunohistochemistry.

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Fig. 1. Peripheral blood absolute counts of total (A) CD3+ T lymphocytes, (B) CD20+ B lymphocytes, (C) CD3+ T lymphocytes at 2 weeks, and (D) WBC during and following antibody treatment.

Fig. 2.

Peripheral LN depletion. A. % T cells in lymph node before depletion and on day 2 after the last dose of depleting reagent with either CD3 IT or rabbit ATG. B. Typical lymph node tissue before treatment and 2 days after the last dose of CD3 immunotoxin treatment (B3511), stained for CD3 (brown) and CD20 (blue). Follicles, paracortical regions, and medullary cords show marked CD3 depletion with CD3 immunotoxin. Typical lymph node tissue before treatment and 2 days after the last dose of rabbit ATG treatment (B6311), stained for CD3 (brown) and CD20 (blue). Rabbit ATG results in partial CD3 depletion.

Table 1

Experimental design.

Treatment	Animal
CD3 immunotoxin – 50 µg/kg (1 dose of 50 µg/kg IV)	B0511
CD3 immunotoxin – 200 µg/kg (8 doses of 25 µg/kg BID IV)	B0411
	B3511
	B6111
Rabbit ATG – 40 mg/kg (2 doses of 20 mg/kg/day IV)	B3411
	B6311