Transplant Immunology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

A reliable method for avoiding false negative results with Luminex single antigen beads; evidence of the prozone effect

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 10 April 2016
Accepted 15 April 2016
Available online xxxx

Keywords:
HLA antibody
Single antigen bead assay
False negative
Prozone
Donor specific antibody

ABSTRACT

Luminex single antigen bead (SAB) assays have become an essential tool in monitoring the status of antibody to the Human Leucocyte Antigen (HLA) of patients both before and after transplantation. In addition SAB data is used to aid risk stratification to assess immunological risk of humoral rejection in solid organ transplantation (CTAG/BTAG guidelines) [1]. Increasingly laboratories are reporting false negative results at high antibody titre due to a prozone effect.

Here we report a case study where the prozone effect led to a false negative antibody result that could have resulted in adverse outcome. We describe a method to reliably remove the prozone effect through heat inactivation and the addition of Ethylenediaminetetraacetic acid (EDTA) to the Luminex wash buffer.

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1. Introduction

The detection and identification of antibody to human HLA is performed pre-transplant where it is vital to avoid HLA mismatches that may lead to acute or hyperacute antibody mediated rejection (AMR) [1,2]; and after transplantation to monitor donor specific antibody (DSA) in order to predict or diagnose AMR [3,4].

Technological advances, including the introduction of solid phase assays, have improved the sensitivity and specificity of the diagnostic tests for HLA antibodies. Luminex (X-map technology) is considered to be the most sensitive of these assays currently available [5–7].

SAB enable highly specific and semi-quantitative HLA antibody detection [5]. Whilst there is no linear association between SAB data and traditional crossmatching results, specific antibody levels around 6000 median fluorescence intensity (MFI) correlate with a positive flow cytometric crossmatch (FCXM); and a DSA above 10,000 MFI is likely to demonstrate a positive complement dependent cytotoxic (CDC) crossmatch result [6].

Although highly sensitive, Luminex SAB testing has been reported to give false negative results despite high antibody titres. This is known as the "hook" or prozone effect and has been attributed to immunoglobulin M (IgM) antibodies or complement components interfering with assay dynamics [7–10]. Schnaidt et al. [9] have shown that pre-treatment of serum with dithiothreitol (DTT), Ethylenediaminetetraacetic acid (EDTA), or standard heat inactivation at 56 °C for 30 min [11,12] can abrogate the prozone effect and unmask potentially clinically significant HLA antibodies.

We present an interesting case of acute AMR following renal transplantation where we sought to define more fully the characteristics of the prozone effect. We have used serum dilution and re-addition of fresh frozen serum following heat inactivation to demonstrate that the effect is likely to be due to steric hindrance involving complement. Following this we describe a validated and reliable laboratory method which we developed to routinely unmask these HLA antibodies. This method has led to the discovery of three further patients demonstrating this prozone effect.

2. Case study

A 22-year-old male with immunoglobulin A (IgA) nephropathy received a pre-emptive transplant from his mother, the HLA mismatches included; A2, B18, Cw7, DR11, DR52, and DQ7.

He received methylprednisolone and basiliximab induction, followed by maintenance immunosuppression with tacrolimus, mycophenolic acid and prednisolone.

Although his graft function remained good (serum creatinine around 120 μmol/L, range 107–129 μmol/L), his attendance at transplant follow

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http://dx.doi.org/10.1016/j.trim.2016.04.002
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up clinics was sporadic and clinicians raised concerns about non-adherence to immunosuppressive medication. Three years after transplantation his follow up lapsed for a six month period; he subsequently presented with a raised serum creatinine of 241 μmol/L. Tacrolimus level was undetectable and the patient confirmed non-adherence to medication including discontinuing steroid therapy since his last clinic attendance six months earlier.

Transplant kidney biopsy demonstrated severe scarring with 13 out of the 18 sampled glomeruli already sclerosed. In addition to features of Type 1a acute cellular rejection, there were focal areas of haemorrhage and interstitial oedema, and peritubular capillaritis with C4d deposition – features consistent with AMR. Immunofluorescence showed granular mesangial deposits of IgA with C3 suggestive of recurrent disease; however the magnitude of this was estimated to have made only a small contribution to the deterioration in graft function.

HLA antibody detection using a standard Luminex SAB test was negative for HLA-Class II DSA and demonstrated only low levels of donor specific HLA-A2 at 3200 MFI. In view of the clinical context and the histological findings, heat inactivation was undertaken to remove the influence of complement in the assay. These techniques are described in the materials and methods section below. Analysis of the HLA-A2 DSA was largely unchanged at 4800 MFI but HLA-Class II SAB revealed >20,000 MFI antibodies against a defined group of HLA DQ antigens. Further investigation suggested the antibody was against a highly immunogenic 41GR3 epitope as described by Duquesnoy et al [13,14], formed around a glycine amino acid at position 40 of the DQ alpha chain [15].

In addition to restarting triple immunosuppression, he received treatment for cellular rejection and AMR, and for recurrent IgA disease (six month treatment with high dose oral prednisolone). AMR treatment included 14 sessions of therapeutic plasma exchange and seven sessions of double-filtration plasmapheresis (DFPP) over 6 weeks. Whilst the HLA-A2 DSA disappeared almost immediately, the HLA-DQ7 (41GR3) specific antibody revealed by heat inactivation remained unchanged at around 22,000 MFI and plasma exchange treatment was therefore discontinued. Unfortunately, graft function has continued to decline and with a current creatinine over 500 μmol/L the patient is heading towards dialysis.

3. Materials and methods

As the prozone effect was not seen with the class I HLA A2 antibody after heat inactivation or with EDTA, the prozone phenomenon was only investigated with respect to class II SAB.

3.1. Donor specific antibody detection

Our laboratory's original standard method for HLA antibody definition using SAB (One Lambda, Canoga Park, CA) is described briefly as follows: 2 μl beads + 10 μl serum were mixed and incubated together at room temperature for 30 min. Samples were washed three times with Luminex wash buffer and centrifuged at 1750 g for 5 min between the washes. 100 μl of PE conjugated antihuman IgG at working concentration was added and the samples incubated in the dark at room temperature for 30 min. Samples were washed twice as before and re-suspended in 80 μl of wash buffer. Fluorescence was measured using the Qiagen liquichip 200 Luminex analyser and analysed using 'HLA Fusion' software version 3.2 (One Lambda, Canoga Park, CA).

3.2. Heat inactivation

Heat inactivation was performed by pre-treating the serum at 56 °C for 30 min in a water bath. The sample was then processed following the method as described above.

This patient's serum was tested using the following techniques:

3.3. Serum dilution

Aliquots of the non-heat treated sample were diluted 1/10 and 1/50 in Luminex wash buffer (One Lambda, Canoga Park, CA) and tested as above for HLA class II antibodies.

3.4. Addition of fresh frozen serum

A further aliquot of the heat-treated sample was mixed with an equal volume of serum from an unsensitised consenting male volunteer (the serum had been prepared previously and stored immediately at −80 °C). Aliquots of both the heat treated sample and the initial non-heat treated sample were diluted 1:1 in wash buffer to control for any dilution effect of the addition of normal serum.

All samples were processed in accordance with the standard method and analysed for HLA-Class II antibodies.

3.5. Addition of EDTA to the wash buffer

Unpublished data from other laboratories has shown that heat inactivation can increase the negative control bead values in some samples, reducing the positive:negative ratio and leading to test failure (defined by the manufacturer as a positive value of at least 500 MFI and positive:negative ratio >2). EDTA has been shown to reduce non-specific binding in other solid phase assays [16] and in a final test we validated the effect of heat inactivation coupled with the addition of 4.5 mM di-sodium EDTA to the wash buffer on 15 samples from both sensitised and unsensitised individuals.

4. Results

4.1. Serum dilution

The sample appeared negative for the DQ7 DSA prior to treatment but was positive for DQA1*04 (DQB1*02 and DQB1*04). Dilution of the sample 1:10 in wash buffer revealed a weak DQ7 DSA (DQA1*05, DQB1*03:01, 6000 MFI) along with weak antibody against DQA1*05:01, DQB1*02:01 and DQA1*06:01, DQB1*03:01. All of these dimers, including those with the DQA1*04:01 alleles, possess glycine at position 40 of the DQ alpha chain [15] (eplet 41GR3 [13]). At 1:50 dilution the sample became strongly positive for all heterodimers possessing this epitope (see Fig. 1). Note that HLA DQA1*04:01 antigen density on Luminex beads has been shown elsewhere to be significantly lower than DQA1*05 or DQA1*06 and may explain the reduced steric hindrance at higher dilutions (see discussion and reference [14]).

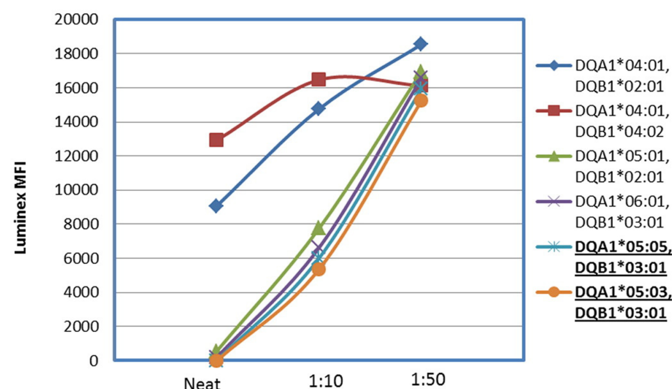

Fig. 1. Dilution of serum 1:10 and 1:50 in Luminex wash buffer reveals the presence of antibody against the 41GR3 eplet. (Luminex MFI values not adjusted for dilution of the sample). Sensitising DSA highlighted in bold. See text for comments on reduced prozone in DQA1*04:01 beads.

Please cite this article as: B.S. Carey, et al., A reliable method for avoiding false negative results with Luminex single antigen beads; evidence of the prozone effect, Transpl Immunol (2016), http://dx.doi.org/10.1016/j.trim.2016.04.002

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