Human Immunology 72 (2011) 849-858

Contents lists available at SciVerse ScienceDirect

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

Novel C1q assay reveals a clinically relevant subset of human leukocyte antigen antibodies independent of immunoglobulin G strength on single antigen beads

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ARTICLE INFO

Article history:
Received 21 April 2011
Accepted 5 July 2011
Available online 18 July 2011

Keywords:
HLA antibodies
Transplantation
Antibody-mediated rejection
Complement
Donor-specific antibodies.

ABSTRACT

It has been known for 40 years that cytotoxic human leukocyte antigen (HLA) antibodies are associated with graft rejection. However, the complement-dependent cytotoxicity assay (CDC) used to define these clinically deleterious antibodies suffers from a lack of sensitivity and specificity. Recently, methods exploiting immunoglobulin G (IgG) antibody binding to HLA single antigen beads (SAB) have overcome sensitivity and specificity drawbacks but introduced a new dilemma: which of the much broader set of antibodies defined by these methods are clinically relevant. To address this, we developed a complement-fixing C1q assay on the HLA SAB that combines sensitivity, specificity, and functional potential into one assay. We compared the CDC, IgG, and C1q assays on 96 sera having 2,118 defined antibodies and determined that CDC detects only 19% of complement-fixing antibodies detected by C1q, whereas C1q detects only 47% of antibodies detected by IgG. In the same patient, there is no predictability by IgG mean fluorescence intensity (MFI) as to which of the antibodies will bind C1q because fixation is independent of MFI values. In 3 clinical studies, $C1q^+$ antibodies appear to be more highly correlated than those detected by IgG alone for antibody-mediated rejection in hearts as well as for kidney transplant glomerulopathy and graft failure.

1. Introduction

The search for a clinically relevant assay to predict antibody-mediated graft rejection (AMR) has been ongoing since 1969 when Patel and Terasaki demonstrated a highly significant (p = 10e-28) but not perfect correlation between a positive crossmatch and hyperacute or accelerated acute graft rejection in kidney transplants using a complement-dependent cytotoxicity (CDC) assay [1]. Newer antibody detection assays, especially those using human leukocyte antigen (HLA) single antigen beads (SAB) on a Luminex (LMX) platform, are highly sensitive and specific for the target antigen but less clinically predictive, with successful transplantation despite moderately strong pretransplant donor-specific immunoglobulin G (IgG) antibodies (DSA) as measured by mean fluorescence intensity (MFI) and negative flow crossmatches (FXM) [2]. The LMX–C4d assay developed to detect complement-fixing antibodies involved in AMR demonstrated a better correlation than CDC with rejection and clinical outcome in heart [3] and kidney [4,5] transplant patients, but has very low sensitivity (MFI range ~ 500–3,500) and limited access to the optimal human serum as a source of complement. Thus, a highly sensitive and specific assay predictive of clinical outcomes still does not exist. Because complement is involved in AMR and AMR portends poorer graft outcome [6–9], an assay distinguishing complement-fixing from non-complement-fixing DSA with high sensitivity and specificity clearly would be useful and lead to a better understanding of the effects of these antibodies on graft outcome. Although both types of antibody may be clinically relevant (*e.g.*, antibody capable of early AMR or graft dysfunction/rejection vs antibody leading to chronic rejection or accommodation [10–13]), the determination of immediate risk at the time of transplant remains a critical clinical need. This is particularly true for highly sensitized patients undergoing desensitization or thoracic transplant candidates where, respectively, prospective crossmatches cannot always be relied upon or performed.

Currently, assessment of rejection risk based on preformed or *de novo* DSA is based on the LMX–IgG MFI strength and FXM channel shifts. Widespread debate exists over the "right" MFI cutoff value for calling an antibody positive or for correlation with FXM results. There is no consensus and each center determines its own threshold consistent with its transplant practices for identifying clinically relevant antibodies, listing "avoids" pretransplant (for virtual crossmatching), or identifying DSA posttransplant requiring therapeutic intervention. High MFI antibodies are considered detrimental [14] but unfortunately, there is a lack of good correlation between DSA MFI and FXM strength with individual or cumulative MFI values [15–17] and values >10,000 can yield negative FXMs (D. Tyan, unpublished observations). Conversely, some have suggested that low MFI values must be "summed" to reach a cumulative MFI value high enough to obtain a correlation with the FXM results

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0198-8859/11/$32.00 - see front matter
doi:10.1016/j.humimm.2011.07.001

[18–20]. Our data suggest instead that the complement-fixing ability of the antibody, irrespective of IgG MFI strength, is a key component of clinical outcome.

Here we present the details of a novel, highly sensitive, and specific antibody assay using the HLA SAB that distinguishes only those antibodies capable of binding the first component of complement, C1q. The $C1q^+$ DSA are highly correlated with rejection and graft failure in solid organ transplants [21,22]. These clinically relevant $C1q^+$ antibodies are independent of IgG MFI strength, suggesting that finding the right or "standardized" cutoff using only the LMX–IgG assay is potentially misdirected.

2. Subjects and methods

2.1. Validation samples

One hundred fifteen samples were used for validation, including 22 male, nontransfused, AB^-, normal blood donor sera ("AB neg"), 20 well-characterized HLA reagent typing sera, and 6 external proficiency sera as negative and positive controls, together with 67 serum samples remaining from clinical testing of solid organ transplant patients. Ninety-six of these sera, positive for 2,118 antibody specificities, were tested by CDC and then by LMX–IgG and LMX–C1q, in parallel, for HLA class I and II. Studies received Stanford Institutional Review Board approval (Nos. 15381/6208, 16996/4947).

2.2. CDC Assay

Cytotoxic assays for class I and II antibodies were performed using 60 well-frozen cell trays (LCT, One Lambda, Inc., Canoga Park, CA) by an extended complement incubation technique and/or by antihuman globulin CDC. One microliter of serum/well was added and incubated for 30 minutes (22°C-T cells, 37°C-B cells) followed by incubation with 5 μl prescreened rabbit complement for 90 minutes (B) or 120 minutes (T) at 22°C. Cells were stained with acridine orange and scored visually using the standard CDC scale (>20% cell death over background = positive).

2.3. LMX–IgG assay

The LMX–IgG assay was performed using SAB kits (LABScreen, One Lambda, Inc.) according to the manufacturer's directions and analyzed on a Luminex (LABScan 100) platform. Each bead is uniquely color coded and coated with a different purified, single HLA class I (n = 97) or class II (n = 92) antigen/allele. Data were analyzed using Visual and Fusion software (One Lambda, Inc.) and interpretations made using normalized (baseline) MFI values. Cutoffs for positive reactions ("true" antibodies) as well as "possible" antibodies were greater than 1,000 and 500-999 MFI, respectively. This assay detects all IgG binding antibodies irrespective of their complement-fixing ability.

2.4. LMX–IgM assay

The same procedure was followed as for the LMX–IgG assay except PE-labeled antihuman IgM second-step antibody (Jackson ImmunoResearch, West Grove, PA) was used instead to detect IgM antibodies. "Normalized" MFI values generated by the same software were used for analysis with similar cutoff values. This assay detects binding antibodies of the IgM isotype.

2.5. LMX–C1q and C1q spiking assays

2.5.1. LMX–C1q

Twenty microliters of serum were incubated with 2.5 μl of LabScreen SAB for 20 minutes at room temperature (RT) and then incubated with 10 μL of custom labeled (BioLegend, San Diego, CA) PE-conjugated sheep antihuman C1q (Biodesign International, Meridian Life Sciences, Inc., Saco, ME) for 20 minutes at RT, washed twice with 200 μl LMX wash buffer, and acquired on the LMX. This assay used autologous C1q. Data were analyzed using HLA Visual software and "Raw" MFI values. Raw values were arranged from highest to lowest MFI and a positive LMX–C1q antibody was assigned when the MFI was >75 MFI over background and the next lower MFI bead and at least twice the MFI of the AB^- control for that individual bead. Negative control sera in this assay are <44 and the negative control bead <10 MFI. This indirect immunofluorescence assay detects all C1q binding antibodies using autologous complement.

2.5.2. LMX–C1q spiking

The same method as for LMX–C1q was used except that sera were first decomplemented (56°C × 30 minutes) and then spiked with 150 μg/mL purified human C1q (hC1q; Sigma) to introduce uniformity and adequate C1q concentrations into all specimens. MFI cutoffs were adjusted to >300 MFI over background and the next lower MFI bead (>30–300% over background). Negative control values typically remain less than 10 MFI.

2.6. C1q depletion and spiking assay

To confirm the essential role of C1q in the assay, rule out other reaction mechanisms, and determine the optimal concentration of C1q, 1 AB^- and 3 well-characterized positive HLA sera were depleted of complement by treating at 56°C for 30 minutes to inactivate complement and then made replete with increasing concentrations of exogenous purified hC1q ranging from 1 to 200 μg/mL and tested by LMX–C1q.

2.7. IgG depletion and spiking assay

To confirm reactions were IgG dependent, separate aliquots of the same sera used for the C1q depletion and spiking assay were depleted of IgG by a protein G affinity technique. Briefly, 150 μl of serum was loaded onto a column containing 0.3 ml dried protein G matrix (Protein G Sepharose 4 Fast Flow; GE Healthcare, Thermo Fisher Scientific, Inc, Waltham, MA) and incubated at RT with gentle rotation for 30 minutes. IgG-depleted sera were collected and complete depletion was confirmed by sensitive IgG enzyme-linked immunosorbent assay. IgG fractions from each positive and negative serum were then eluted from the protein G beads, concentrated 10×, and added back to the original source serum at concentrations ranging from 0.35 μg/mL to 10 mg/mL. Each depleted and reconstituted serum was tested by LMX–C1q.

3. Results

3.1. C1q assay

The principles and differences between the LMX–C1q and LMX–IgG assays are illustrated in Fig. 1. For C1q, the 22 AB^- control sera yielded values between 3 and 44 MFI on class I or II beads. HLA^+ control sera yielded MFIs ranging up to 32,286 (Fig. 2), with values for the negative control bead (no antigen) usually <10 MFI. The mean coefficient of variation for intra- and interassay comparisons was 16.8 and 25.7%, respectively. LMX–C1q (without spiking) accurately detected complement-fixing antibody in the oldest HLA^+ serum (frozen at -70°C since 1974), suggesting that serum C1q is very stable and remains functional.

3.2. C1q depletion and spiking assay

IgG^+ sera that underwent complement inactivation in the autologous C1q assay demonstrated no positive C1q signal with background MFIs on all SAB < 100 (Fig. 3). Adding back increasing amounts of purified hC1q increasingly revealed the previously defined cytotoxic/$C1q^+$ specificities, whereas the AB control serum remained negative with similar spiking even when the hC1q concentration reached 200 μg/mL. The minimum hC1q concentration needed to detect a positive specificity was 25 μg/mL, 6 times less