Nature and Clonality of the Fluoresceinated Secondary Antibody in Luminex Multiplex Bead Assays Are Critical Factors for Reliable Monitoring of Serum HLA Antibody Levels in Patients for Donor Organ Selection, Desensitization Therapy, and Assessment of the Risk for Graft Loss

Mepur H. Ravindranath, Vadim Jucaud, Nubia Banuelos, Matthew J. Everly, Junchao Cai, Anh Nguyen and Paul I. Terasaki

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Nature and Clonality of the Fluoresceinated Secondary Antibody in Luminex Multiplex Bead Assays Are Critical Factors for Reliable Monitoring of Serum HLA Antibody Levels in Patients for Donor Organ Selection, Desensitization Therapy, and Assessment of the Risk for Graft Loss

Mepur H. Ravindranath, Vadim Jucaud, Nubia Banuelos, Matthew J. Everly, Junchao Cai, Anh Nguyen, and Paul I. Terasaki

Luminex multiplex immunoassays enable simultaneous monitoring of Abs against multiple Ags in autoimmune, inflammatory, and infectious diseases. The assays are used extensively to monitor anti-HLA Abs in transplant patients for donor organ selection, desensitization, and assessing the risk for graft rejection. To monitor IgG Abs, fluoresceinated IgG constant H chain–binding polyclonal F(ab')2 (IgHPolyFab) is used as the fluoresceinated secondary Ab (2nd-Ab), whereas IgG subclasses are monitored with Fc-specific monoclonal whole IgG (FcMonoIgG). The fluorescent signal from the 2nd-Ab is measured as mean fluorescence intensity (MFI). When IgHPolyFab is used, the signal is amplified as a result of the binding of multiple polyclonal Fabs to the C region of primary IgH. The reliability of such amplification for Ab measurements was not validated, nor were MFIs compared with 1:1 binding of FcMonoIgG to primary Abs. Comparing the MFIs of anti-HLA Abs obtained with IgHPolyFab and FcMonoIgG against normal human sera, IVIg, and allograft recipients’ sera, it was observed that the number of HLA-Abs was notably higher when 1:1 binding of FcMonoIgG was used, the signal is amplified as a result of the binding of multiple polyclonal Fabs to the C region of primary IgH. The reliability of such amplification for Ab measurements was not validated, nor were MFIs compared with 1:1 binding of FcMonoIgG to primary Abs. Comparing the MFIs of anti-HLA Abs obtained with IgHPolyFab and FcMonoIgG against normal human sera, IVIg, and allograft recipients’ sera, it was observed that the number of HLA-Abs was notably higher with IgHPolyFab than with FcMonoIgG. The MFIs of anti-HLA Abs also remained higher with IgHPolyFab in the normal sera and in IVIg, but the reverse was true when the autologous and allogeneic IgG concentrations were augmented in allograft recipients. Indeed, MFIs of the de novo allo-HLA Abs were markedly higher with FcMonoIgG than with IgHPolyFab. Serum titration established the superiority of FcMonoIgG for monitoring MFIs of de novo allo-HLA Abs in allograft recipients. Avoiding false amplifications of the number and MFIs of anti-HLA IgG with FcMonoIgG may minimize immunosuppressive therapies, maximize the number of donors for patients waiting for allografts, and enable better prediction of graft rejection. The Journal of Immunology, 2017, 198: 4524–4538.

The development of Luminex Multiplex Single-Antigen Bead (SAB) Assays was a significant step toward sensitive and specific detection of Abs against multiple Ags simultaneously in sera of patients with autoimmune diseases (1–4), cancer (5), transplantation (6, 7), and other disease conditions (8). The Luminex SAB assays involve Abs immobilized onto spherical beads in suspension and the use of these immobilized beads to capture serum Abs against the coated Ags. The captured Ab is identified by a fluorescence-labeled fluoresceinated secondary Ab (2nd-Ab)–based detection system (9, 10). The detection system in Luminex assays involves dual lasers: one specifically identifies the Ag-coated colored microbeads, and the other determines the magnitude of the signal derived from the 2nd-Ab. The levels of Abs are assessed as mean fluorescent intensity (MFI). MFI has become a critical guide for monitoring anti–HLA-IgG Abs in patients waiting for donor organ selection, desensitization therapy, and to assess the risk for acute and chronic humoral rejection (6, 7). Because titration of HLA Abs with Luminex SAB assay is cost prohibitive, the MFI derived from the fluorescent signal of sera at a particular dilution is considered suitable to predict the level of primary-Abs bound to Ags on the beads (6, 7, 9, 10).

Consistency and specificity of MFI values of serum IgG Abs may be affected by extrinsic factors like IgM (11, 12) and aggregation of serum IgG, with or without other serum proteins (13–17) when
neat or low-dilution sera are used (11), and by intrinsic factors, such as the Luminex platform and the vendor-supplied SAB analytical reagents (7, 14). Therefore, quality control maximizing the specificity and reliability of MFI of serum IgG requires serious and step-wise validation of the Ag-coated beads and the detection system. Recently, we have examined the confrontational variants of the individual HLA coated on beads that could affect the specificity of the IgG binding to native HLAs (HLA trimers) (18, 19). This investigation focuses on the influence of 2nd-Ab, a minor and much-ignored reagent that is critical for determining MFI of serum anti-HLA IgG. To the best of our knowledge, almost all of the published reports do not specify the nature and/or clonality of the 2nd-Ab but instead state that they used the 2nd-Ab “according to the manufacturer’s instructions” (20–24).

The conventional SAB assay uses an IgG constant H chain (HC)-binding polyclonal F(ab’)_2 (IgHPolyFab) as the 2nd-Ab provided by the vendor. IgHPolyFab binds to the “constant region” of IgH (25) and recognizes the IgG bound to HLA-coated SAB. There is no doubt that IgHPolyFab is a valuable reagent for the resolute localization of surface Ags on cells and tissue sections and offers “...increased sensitivity through the signal amplification that occurs as multiple secondary antibodies bind to a single primary antibody” (26). Although such signal amplification is useful in flow cross-matching and histopathology, it would boost, amplify, or exaggerate MFI values in SAB assays.

In contrast, IgG subclasses are monitored with PE-conjugated Fc-specific monoclonal whole IgG (FcMonoIgG) (27). However, FcMonoIgG hitherto has not been employed to monitor MFI of anti–HLA-IgG nor has any empirical comparison of MFI been hitherto has not been employed to monitor MFI of anti–HLA-IgG nor has any empirical comparison of MFI been obtained with FcMonoIgG. In essence, MFI obtained with IgHPolyFab is a result of signal amplification due to its binding to multiple epitopes on the IgH of a single IgG, whereas MFI obtained with FcMonoIgG is a result of its binding to one Fc-epitope of a single IgG. Therefore, we hypothesize that MFI obtained with FcMonoIgG, rather than IgHPolyFab, measures the reliable proportion of primary Abs bound to Ag-coated beads, without amplification or exaggeration of the fluorescence signal and while minimizing false positivity. The hypothesis is tested in a Luminex SAB assay by comparing MFI obtained with the conventionally used IgHPolyFab versus FcMonoIgG, using the sera of “nontransplant” donors, pre- and posttransplant patients, and IVIg.

Materials and Methods

Sera of healthy and nonhealthy males and females

Healthy (n = 11) and nonhealthy (n = 5) [endometriosis; n = 1, leukemia; n = 1, autoimmune disease; n = 1, squamous cell carcinoma; n = 1, and dialysis; n = 2, repeat bleed] volunteers provided sera after informed consent. Sera were consistently used after 1:10 dilution or after purification of IgG (final dilution 1:10) to avoid interference with IgM or other proteins in the analysis. Serum IgGs were purified using Protein G columns (Thermo Fisher, Rockford, IL), and the three eluates (E1, E2, E3) of purified IgG were obtained as described earlier (28). In most cases, the sera were collected soon after the blood was drawn, the sera IgG were immediately purified, and SAB assays were performed simultaneously on the sera and purified IgG.

Sources of the therapeutic preparations of IVIg

The IVIg preparations examined earlier (29, 30) were used: GamaSTAN S/D (15–18%, Lot 26NHCVI), GAMUNEX-C (10%, Lots 26NKLG1 and 26NKLK1; all from Talecris Biotherapeutics, Research Triangle Park, NC), and Octagam (6%, Lot A913A8431; Octapharma Pharmazeutika, Lachen, Switzerland). All IVIg was serially diluted with 1× PBS (pH 7.2).

Sera of renal allograft recipients

Sera were used from 10 living donor kidney transplant recipients who were transplanted in 2008. Protocol and consent forms were approved by their institute’s review board. None of the patients had been transplanted previously. Pre- and posttransplant treatment protocols are described elsewhere (31–34). Pretransplant serotyping was performed using Lambda Monoclonal Trays (One Lambda, Canoga Park, CA). When the donor-specific Abs (DSAs) reacted with highest MFI to an Ag in the donor’s serotype Ag group, that Ag is recognized as the molecular type of the donor. Pretransfusion sera, posttransfusion but pretransplant sera, and posttransplant sera were obtained from each patient and frozen. The sera were diluted (1:10) or titrated further to examine pre-existing and de novo DSAs and non-DSAs.

Luminex multiplex SAB assay

HLA-I and HLA-II IgG reactivity were analyzed using SAB, and data were acquired with a dual-laser flow cytometry Luminex-xMAP (LABScanTM 100; One Lambda) (5, 28–30). The single HLA-Ia and HLA-II Ags in LS1A04-Lot (007) (for HLA-Ia) contain 31 HLA-A, 50 HLA-B, and 16 HLA-Cw molecules, whereas those in LS2. A01009 (Lot 9) (for HLA-II) contain 29 HLA-DRB1, 7 HLA-DRB3, 4, 5, 29 HLA-DQ, and 26 HLA-DP molecules. The SAB assay includes built-in control beads, coated with human IgG (positive control) or albumin (human or bovine; negative control). The assay involved incubation of 20 μl of sample with 2 μl of beads for 30 min at room temperature, on a shaker. The beads were then washed three times with LABScreen Wash Buffer. The Ab binding to beads was monitored using different kinds of PE-conjugated 2nd-Abs, by incubating the 2nd-Ab (50 μl at 5 μg/ml) for 30 min at room temperature on a shaker. After washing, the beads were suspended in 1× PBS before acquisition on the Luminex. At least 100 beads were counted for each Ag, and the IgG reactivity was recorded as trimmed MFI after normalizing the MFI against the negative control (bead #1) and then with PBS (negative control sample).

We have recently recognized the structural variants of HLA class I molecules coated onto the beads (18, 19). Not all Ags are of same density on beads nor is the density of a single Ag the same on all beads. Therefore, ≥100 beads are measured for every Ag to obtain a mean and SD. Mean is always greater than 2 SD. The same beads were used when comparing the efficacy of two 2nd-Abs.

Nature and clonality of PE-conjugated 2nd-Abs

The three PE-conjugated 2nd-Abs used in this study differ with regard to their nature, clonality and sources, as described below.

IgHPolyFab. Human IgG constant HC-binding affini-pure polyclonal F(ab’)_2, 0.5 mg/ml in PBS (pH 7.6), was purchased from One Lambda (cat. no. LS-82). The label on the box of vials describes the product as “PE-conjugated goat-anti-human IgG, R-phycocerythrin-conjugated affini-pure F(ab’)_2, goat X-human IgG 1 ml (100×),” indicating that it is an HC-binding polyclonal F(ab’)_2.
Table I. Disparity in the number of HLA Ags reactive to primary IgG in native sera (1:10) and in purified IgG eluates (E2 and E3) from sera (1:10) when monitored with the 2nd-Abs IgHPolyFab and FcMonoIgG

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Larger number of primary HLA Abs recognized by either IgHPolyFab or FcMonoIgG is shown in bold type.

*Abs per locus.
Table II. Disparity in the MFI of anti–HLA-I reactivity of the native sera and purified IgG eluates (E2 and E3) (1:10) of volunteers (F07 and F01) when monitored with the 2nd-Abs IgHPolyFab and FcMonoIgG

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<td>2579</td>
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<td>3070*</td>
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</table>

MFI in bold: IgHPolyFab or FcMonoIgG is higher than the other. Alleles in bold and italics are found only with IgHPolyFab. MFI with IgHPolyFab is always higher (MFI in bold) than that for FcMonoIgG. If no MFI value is given, it is <500.

*aHigher MFI with FcMonoIgG.
Table III. Disparity in the MFI of anti–HLA-II reactivity of the native sera and purified IgG eluates (E2 and E3) (1:10) of volunteers (F07 and F01) when monitored with the 2nd-Abs IgHPolyFab and FcMonoIgG

<table>
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<tr>
<th>HLA-DR</th>
<th>IgHPolyFab S (1:10)</th>
<th>IgHPolyFab E2</th>
<th>IgHPolyFab E3</th>
<th>FcMonoIgG S (1:10)</th>
<th>FcMonoIgG E2</th>
<th>FcMonoIgG E3</th>
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<tbody>
<tr>
<td>DRB1*03:02</td>
<td>503 1,564 1084</td>
<td>746 1,176(^a)</td>
<td>1,753 2,070 1,090</td>
<td>1,600 1,045 861</td>
<td>DQA1<em>02:01/DQB1</em>03:01</td>
<td>DPA1<em>01:03/DPB1</em>11:01</td>
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<tr>
<td>DRB1*13:01</td>
<td>583 1,555 1,426</td>
<td>1,243 1,426</td>
<td>4,938(^b) 1,998</td>
<td>3,184</td>
<td>DPA1<em>01:03/DPB1</em>19:01</td>
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</tr>
<tr>
<td>DRB1*14:02</td>
<td>631 2,915 2,540</td>
<td>1,149</td>
<td>1,622 799</td>
<td>DPA1<em>01:05/DPB1</em>13:01</td>
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<tr>
<td>DRB3*03:01</td>
<td>1,768 1,845</td>
<td>2,809 2,701</td>
<td>1,189</td>
<td>1,636 817</td>
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<td>F07</td>
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<td>503 1,564 1084</td>
<td>746 1,176(^a)</td>
<td>1,753 2,070 1,090</td>
<td>1,600 1,045 861</td>
<td>DQA1<em>02:01/DQB1</em>03:01</td>
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<tr>
<td>DRB1*13:01</td>
<td>583 1,555 1,426</td>
<td>1,243 1,426</td>
<td>4,938(^b) 1,998</td>
<td>3,184</td>
<td>DPA1<em>01:03/DPB1</em>11:01</td>
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<td>1,149</td>
<td>1,622 799</td>
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<td>1,636 817</td>
<td>DPA1<em>02:02/DPB1</em>13:01</td>
<td></td>
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</table>

**MFI in bold:** IgHPolyFab or FcMonoIgG is higher than the other. Alleles in bold and italics are found only with IgHPolyFab. MFI with IgHPolyFab is always higher (MFI in bold) than with FcMonoIgG. If no MFI value is given, it is <500.

\(^a\)HLA-DP*: no reactivities are seen for S (1:10) with either of the 2nd-Abs.

\(^b\)Higher MFI with FcMonoIgG.
FcMonoIgG. Human IgG Fc-specific mouse monoclonal IgG, 0.5 mg of purified IgG in 1 ml of borate buffered saline (pH 8.2), was purchased from SouthernBiotech (Birmingham, AL). The product insert states that Fc-specific IgG is derived from clone JDC-10 and reacts specifically with human/rhesus/chimpanzee IgG Fc (molecular mass 150 kDa).

FcPolyIgG. Human IgG Fc fragment HC-binding affini-pure polyclonal IgG, 0.5 mg/ml in PBS (pH 7.6), was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA; code #109-115-099). The product insert states that PE-conjugated affini-pure Goat anti-Human IgG reacts with the Fc portion of IgG but not with the Fab portion of IgG. It may cross-react with IgGs of other species. This 2nd-Ab was used to analyze the sera of allograft recipients, and the data are presented as a figure.

Statistical analysis

A paired-sample t test (two-tailed) was used to assess the disparity between IgHPolyFab and FcMonoIgG if both of the variables were normally distributed. If the variables were not normally distributed, the Wilcoxon matched-pair signed-rank test (two-tailed) was used for equal variance and the sign test of matched pairs (two-tailed) was used for unequal variance. Statistical analyses were done with STATA-13.

Results

Sera of healthy and nonhealthy volunteers

Disparity in the MFIs of serum IgG binding to albumin-coated negative-control beads between IgHPolyFab and FcMonoIgG. Because negative-control beads are coated with albumin, IgG binding to the beads may signify anti-albumin Abs. Fig. 1 compares anti-albumin IgG MFI in the volunteers’ sera (at 1:10 dilution) obtained with IgHPolyFab and FcMonoIgG. Although these MFIs are considered negative (<500), the objective is to document significant amplification of the fluorescent signals by IgHPolyFab (3-fold for native serum IgG and 7-fold for purified IgG) compared with FcMonoIgG. Fig. 1 illustrates the significant disparity in the MFI between the two 2nd-Ab.

Table IV. Disparity in the number of anti-HLA IgGs in the different therapeutic preparations of IVIg, as recognized by the 2nd-Abs IgHPolyFab and FcMonoIgG

<table>
<thead>
<tr>
<th>Nature of PE–2nd-Ab (0.5 mg/ml)</th>
<th>HLA Loci</th>
<th>IgHPolyFab</th>
<th>FcMonoIgG</th>
<th>Statistical analysis</th>
<th>Paired-sample t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration 10%</td>
<td>HLA-A*</td>
<td>31 (1:20)</td>
<td>16 (1:40)</td>
<td>p² &lt; 0.01</td>
<td>p² = 0.02</td>
</tr>
<tr>
<td>Protein concentration 15–18%</td>
<td>HLA-A*</td>
<td>30 (1:20)</td>
<td>10 (1:40)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Protein concentration 6%</td>
<td>HLA-A*</td>
<td>9 (1:20)</td>
<td>1 (1:40)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paired-sample t test</td>
<td>Protein concentration 10%</td>
<td>DRB*</td>
<td>36 (1:20)</td>
<td>9 (1:40)</td>
<td>p² &lt; 0.0001</td>
</tr>
<tr>
<td>Protein concentration 15–18%</td>
<td>DRB*</td>
<td>36 (1:20)</td>
<td>9 (1:40)</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Protein concentration 6%</td>
<td>DRB*</td>
<td>31 (1:20)</td>
<td>9 (1:40)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Disparity in the serum anti-HLA IgG profiles and MFIs between IgHPolyFab and FcMonoIgG

The details of the disparities observed in the number and MFI of anti-HLA IgG Abs recognized by the two 2nd-Abs in the sera and in the IgG purified from the sera of normal donors are described in the section that follows.

Disparity in the number of anti-HLA IgG Abs recognized by the two 2nd-Abs in sera of normal donors. Table I shows the differences in the number of anti-HLA IgG Abs recognized in the serum IgG with two 2nd-Abs: IgHPolyFab and FcMonoIgG. It further depicts that a larger number of anti-HLA Abs are detected with IgHPolyFab (values in bold type) than with FcMonoIgG. For example, the serum IgG of F01 showed reactivity against 10 HLA-A Ags and 12 HLA-DR Ags with IgHPolyFab, whereas only 7 HLA-A and 5 HLA-DR Ags were recognized with FcMonoIgG. In striking contrast to FcMonoIgG, PE-conjugated 2nd-Ab IgHPolyFab recognizes IgG reacting to HLA-B (M02/M04/M05), HLA-Cw (M04), and HLA-DR (M04/M05) in the sera of normal males. Similarly, it also recognized IgG reactivity against HLA-A (F01), HLA-B (F06), and HLA-DR (F01, F02) in the sera of females, as well as reactivity against HLA-A (M07), HLA-B (M61), HLA-DR (F07/M07), and HLA-DQ (M061A) in the sera of nonhealthy volunteers. The last two rows in the table reveal that the total numbers of anti-HLA Abs recognized with IgHPolyFab are strikingly higher than those for FcMonoIgG. These observations indicate that the results are the consequence of fluorescent signal amplification caused by multiple binding of IgHPolyFab to different epitopes on the C region of HC of the primary Ab. These findings provide proof for possible false positivity with the vendor-recommended 2nd-Ab (i.e., IgHPolyFab).

Disparity in the number of HLA IgG Abs recognized by the two 2nd-Abs in the purified IgG of sera from normal donors. IgG molecules in the neat sera, those at low dilution (<1:10), or those

Table I shows the differences in the number of anti-HLA IgG Abs recognized with IgHPolyFab and FcMonoIgG.
<table>
<thead>
<tr>
<th>IVIg Sources</th>
<th>GAMUNEX-C</th>
<th>GamaSTAN</th>
<th>GAMUNEX-C</th>
<th>GamaSTAN</th>
<th>GAMUNEX-C</th>
<th>GamaSTAN</th>
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</thead>
<tbody>
<tr>
<td>Protein Concentration</td>
<td>10%</td>
<td>15-18%</td>
<td>10%</td>
<td>15-18%</td>
<td>10%</td>
<td>15-18%</td>
</tr>
<tr>
<td>HLA-A</td>
<td>IgHPolyFab</td>
<td>FcMonoIgG</td>
<td>IgHPolyFab</td>
<td>FcMonoIgG</td>
<td>IgHPolyFab</td>
<td>FcMonoIgG</td>
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<td>B*07:02</td>
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<td>537</td>
<td>Cw*01:02</td>
<td>4429</td>
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<tr>
<td>A*02:01</td>
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<td>B*08:01</td>
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<td>Cw*02:02</td>
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</table>

MFI in bold: IgHPolyFab or FcMonoIgG is higher than the other. Alleles in bold and italics are found only with IgHPolyFab. MFI with IgHPolyFab is always higher (MFI in bold) than those with FcMonoIgG. If no MFI value is given, it is <500.

The paired-sample test on the difference between the MFIs of the two 2nd-Abs for HLA-Cw alleles under the column headed GAMUNEX-C is significant at p < 0.0001.

Higher MFI with FcMonoIgG.
Table VI. Disparity in the MFI of HLA-II–reactive Abs in different therapeutic preparations of IVIg (at dilution 1:40) recognized by 2nd-Abs IgHPolyFab and FcMonoIgG

<table>
<thead>
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<th>Protein Concentration</th>
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<th>GAMUNEX-C</th>
<th>GammaSTAN</th>
<th>GAMUNEX-C</th>
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</thead>
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<td>FcMonoIgG</td>
<td>IgHPolyFab</td>
<td>FcMonoIgG</td>
<td>IgHPolyFab</td>
<td>FcMonoIgG</td>
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MFI in bold: IgHPolyFab or FcMonoIgG is higher than the other. Alleles in bold and italics are found only with IgHPolyFab. MFI with IgHPolyFab is always higher (MFI in bold) than that for FcMonoIgG. If no MFI value is given, it is <500.

*Higher MFI with FcMonoIgG.
Table VII. HLA serotyping of male renal transplant patients (Ren) and their respective donors

<table>
<thead>
<tr>
<th>Ren-1 (33 y)</th>
<th>Ren-2 (40 y)</th>
<th>Ren-3 (42 y)</th>
<th>Ren-4 (32 y)</th>
<th>Ren-5 (22 y)</th>
<th>Ren-6 (24 y)</th>
<th>Ren-7 (19 y)</th>
<th>Ren-8 (47 y)</th>
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MFIs of de novo DSAs (±/+/++/+++ and pre-existing Abs (+*/++*/+++*) in pretransfusion and pretransplant sera and the presence of auto-HLA Abs (Φ) in allograft recipients. Numbers in parentheses indicate revised split serotypes. Bold numbers refer to donor HLA types.

*Mismatch.

Table VIII. Disparity in the number of HLA alleles recognized by the 2nd-Abs IgHPolyFab and FcMonoIgG in the Post-Tx sera (IgG) of renal transplant recipients

<table>
<thead>
<tr>
<th>Patient ID for Post-Tx Sera</th>
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<th>No. of Allele-Reactive IgGs in HLA Class II</th>
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<tr>
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Note the higher number of alleles recognized by IgHPolyFab in the SAB assay. FcMonoIgG revealed no such increased number of allo-HLA IgAbs, indicating that the high number of allo-HLA IgAbs observed with IgHPolyFab could due to false positivity. Anti-HLA IgAbs are commonly monitored with IgHPolyFab in clinical transplantation; the data suggest more false positivity when IgHPolyFab is used as the 2nd Ab. Based on the exaggerated MFI with IgHPolyFab, it is often concluded that patients waiting for donor organ are sensitized (abundant allo-HLA Abs). For lowering allo-HLA Abs, desensitization therapy is developed using IVIg or other agents. Bold numbers refer to a higher number of alleles recognized by the particular 2nd-Ab.

Post-Tx, posttransplantation.

Wilcoxon matched-pairs signed-rank test.
subjected to freeze-thaw and/or centrifugation self-aggregate or aggregate with IgM, complement, and other proteins (9–15). The polyclonal IgHPolyFab can easily recognize IgH in these aggregates. IgG eluates purified from the sera revealed a greater diversity of HLA-I and HLA-II reactivity compared with native serum IgG (21, 23). Indeed, once again, the number of reactive Abs is higher with IgHPolyFab than with FcMonoIgG. The last rows in Table I summarize that the number of Abs recognized by the purified IgG in the eluates (E2, E3) is significantly higher with IgHPolyFab than with FcMonoIgG. Because the E2 fraction had a higher concentration of IgG than the E3 fraction, it showed a markedly higher diversity (Table I). However, in rare cases, the anti–HLA-II IgG identified with FcMonoIgG had higher MFI than that obtained with IgHPolyFab, possibly due to higher Ab concentration (see below).

Disparity in the MFI levels of anti-HLA IgG Abs recognized by the two 2nd-Abs in the sera from normal donors. When the MFIs of the native serum and the purified IgG from the respective volunteer’s serum were compared between the two 2nd-Abs, the MFIs are most often higher with IgHPolyFab compared with FcMonoIgG. This was consistently seen in all volunteers. To illustrate this finding, the MFIs obtained in two volunteers are shown in Tables II and III. A conspicuous disparity is observed in the MFIs obtained using the two 2nd-Abs. This difference was apparent among the native sera, as well as the purified IgG (E2 and E3) of sera from F07 and F01. A number of anti–HLA-I (Table II) and anti–HLA-II (Table III) IgGs with positive MFI (>500) are observed only with IgHPolyFab for native and purified IgG (values in bold type). Many of these Abs were not recognized by FcMonoIgG (Tables II, III), suggesting that, as a 2nd-Ab, IgHPolyFab has a propensity to generate false positivity. Infrequently, the MFI of a few anti–HLA IgG with FcMonoIgG were higher than with that of IgHPolyFab, possibly due to greater Ab density in the sera (see Discussion).

Anti–HLA IgG in therapeutic preparations of IVIg

The details of the disparities observed in the number and MFI of anti–HLA IgG Abs recognized by the two 2nd-Abs in the IVIg preparations are described in the section that follows.

Disparity in the number of anti–HLA IgG Abs recognized by the two 2nd-Abs in IVIg. All IVIg preparations showed a wide variety of HLA-I and HLA-II reactivities, with varying MFIs (29, 30, 35). Table IV reveals the striking disparity in the number of anti–HLA-I and anti–HLA-II Abs recognized by IgHPolyFab and FcMonoIgG in the IVIg preparations. Table III shows that the number of anti–HLA IgGs recognized in IVIgs using IgHPolyFab is significantly greater than the number recognized by FcMonoIgG. Of the three IVIg preparations, GAMUNEX-C showed the greatest disparity between IgHPolyFab and FcMonoIgG, possibly as a result of the different protocols used during IVIg purification. Disparity in the MFI levels of anti-HLA IgG in IVIg recognized by the two 2nd-Abs. Such amplification is also evident in the MFIs of anti–HLA-I (Table V) and anti–HLA-II (Table VI) IgGs when IgHPolyFab, but not FcMonoIgG, is used. The MFI signals obtained with IgHPolyFab are consistently higher. When the anti–HLA IgG of IVIg is examined with FcMonoIgG, high MFI profiles are extremely rare. Importantly, the IVIg preparations differed markedly with regard to the profiles of HLA Abs.

Sera IgG of renal allograft recipients

Sera from transplant patients were drawn just before transfusion of donor blood (pretransfusion sera), before transplantation (pretreatment sera), and after transplantation (posttransplant sera). The de novo DSAs or de novo non-DSAs were identified in post-transfusion sera when no or low level of Abs (MFI < 500) were detected in pretransfusion and pretransplant sera. Table VII shows the HLA serotypes of the patients (n = 10) and their donors, as well as a patient (Ren-2) with anti-HLA autoantibodies (anti-DR52). The de novo DSA against HLA-A Ags is found in Ren-6, HLA-B Abs in Ren-3, HLA-Cw Abs in Ren-1 and Ren-5, and HLA-DR Abs in Ren-2, Ren-4, Ren-6, and Ren-9. The de novo non-DSA was prevalent against HLA-DQ in 3 of 10 patients.

Disparity in the number of anti–HLA IgG Abs recognized by the two 2nd-Abs. Table VIII shows that the number of allo–HLA–reactive serum IgGs is notably higher (values in bold type) with IgHPolyFab than with FcMonoIgG in the allograft recipients. A strikingly higher number of HLA-reactive serum IgGs was observed for HLA-B (p<0.02) and HLA-DR (p=0.02). For example, the serum IgG of patient Ren-6 showed higher reactivity to 28 HLA-B, 10 HLA-DRB1, and 11 HLA-DQA DQs with IgHPolyFab, whereas only 19
and FcMonoIgG and non-DSAs (MFI and greater range of MFI for de novo DSAs (dilution) (Fig. 2). At 1:10 dilution of sera, a significantly higher literature and 1:10 dilution) and FcPolyIgG Abs (DSAs) (Fig. 3A) and non-DSAs (Fig. 3B) were also examined with an-

FIGURE 3. Disparity in MFIs for the de novo donor specific anti-HLA Abs recognized by the two 2nd-Abs. Patient Ren-6 had DSAs for donor serotypes A24 (Fig. 4A), DR52 (Fig. 4B), and DQ7 (Fig. 4C). Comparing the MFI profiles of IgHPolyFab and FcMonoIgG after titration of the serum Abs, it is obvious that MFIs of anti-HLA IgG Abs ascertained with FcMonoIgG in the sera of allograft recipients were higher at all dilutions and even at higher dilutions. The serum titration profiles of de novo DSAs against DQ7 (Fig. 4C) clearly reveal differences in anti-HLA IgG MFI levels between IgHPolyFab and FcMonoIgG at identical concentrations. For DQA1*02:01|DQB1*03:01 and DQA1*03:01|DQB1*03:01, MFI of 5000 was reached at a serum dilution of 1:20 for IgHPolyFab, whereas FcMonoIgG reached the same MFI at serum dilutions of 1:200 and 1:400, respectively. Such differences can be seen for other molecular types of DQ7, illustrating that FcMonoIgG is more sensitive than IgHPolyFab for determining MFI, even at higher dilutions of sera. Other DSAs ([A24] Fig. 4A, [DR52] Fig. 4B) illustrate similar differences and the higher sensitivity of FcMonoIgG for establishing MFI levels. Fig. 4D documents MFI obtained by titrating 2nd-Abs. The original concentration of IgHPolyFab and FcMonoIgG was 0.5 mg/ml. Comparing these two 2nd-Abs, one may note that MFI of Abs to DBR3*02:02 determined with IgHPolyFab at 1:20 dilution is comparable to that obtained with FcMonoIgG at 1:80 dilution (Fig. 4D). The same is true for serum Abs reacting to DQ7 (data not shown).

Discussion

MFI obtained using the dual-laser LumineX SAB assay is a measure of the magnitude of the fluorescence signal derived from 2nd-Abs that are bound to primary Abs, which, in turn, are attached to HLA-coated beads. MFIs obtained with a 2nd-Ab will be in direct proportion to the primary Ab, only if the 2nd-Abs bind to the primary Abs in a 1:1 ratio. Such MFIs at a particular dilution of sera can be used for quantitative assessment of Ab concentration.

If unspecified or varying numbers of 2nd-Abs bind to a single primary Ab, it will not be in direct proportion to the level of primary Ab; this can only be used for a qualitative evaluation, particularly when the serum is not titrated. Therefore, the reliability of MFI strictly depends on whether the 2nd-Abs bind to the primary Abs in a 1:1 ratio. Most investigators, including our group, monitoring the humoral response to transplantation use a LumineX multiplex multiarray SAB assay because of its remarkable sensitivity compared with cytotoxicity and flow cross-match assays and customarily use IgHPolyFab as a 2nd-Ab, as recommended by the vendor that supplies the beadsets for the LumineX assays.

The inset in Fig. 1 illustrates binding of multiple polyclonally derived IgHPolyFab to both of the constant domains of IgH, whereas a single molecule of monoclonally derived FcMonoIgG binds to the Fc domain of IgG. The fluorescent signal of FcMonoIgG that binds to the primary Abs on a single bead reflects the density of the primary Abs on a bead, which is measured as MFI. Because the magnitude of the signal derived from IgHPolyFab is due to binding of multiple 2nd-Abs, the number of HLA-reactive Abs is exaggerated, leading to false positivity for many of them. MFI of a particular anti-HLA Ab is obtained after “normalizing” the mean MFI of ~100 beads. To the best of our knowledge, no attempt has been made to empirically validate MFIs obtained with IgHPolyFab and FcMonoIgG. Strangely, almost all publications totally overlooked

FIGURE 3. Disparity in MFIs for the de novo donor specific anti-HLA Abs (DSAs) (A) and de novo non–donor-specific allo-HLA Abs (NDSA) (B) in renal allograft recipients (n = 7) is revealed among three PE-conjugated 2nd-Abs (sera are tested at 1:10 dilution): IgHPolyFab, FcMonoIgG, and FcPolyIgG. Paired-sample $p < 0.0001$ IgHPolyFab versus FcMonoIgG and FcMonoIgG versus FcPolyIgG.

HLA-B, 9 HLA-DRB1, and 9 HLA-DQ Ags are recognized by the monoclonal IgG. Such a striking difference in the number of anti-HLA IgG Abs obtained with the two 2nd-Abs support the contention that the higher HLA reactivity observed with IgHPolyFab as a 2nd-Ab could be due to false positivity.

Disparity in the MFI levels of anti-HLA IgG Abs recognized by the two 2nd-Abs. The MFI of the de novo DSAs and non-DSAs in the renal allograft recipients showed statistically significant disparities between IgHPolyFab (sera tested at 1:3 [as is often done in the literature] and 1:10 dilution) and FcMonoIgG (sera tested at 1:10 dilution) (Fig. 2). At 1:10 dilution of sera, a significantly higher MFI and greater range of MFI for de novo DSAs ($p < 0.0001$) and non-DSAs ($p^2 < 0.0001$) were observed for all patients with FcMonoIgG than with IgHPolyFab. At this dilution, MFI of DSAs (Fig. 3A) and non-DSAs (Fig. 3B) were also examined with an-
the characteristics of the 2nd-Ab as minor features of the multiplex immunoassays; consequently, the importance of the nature and clonality of the 2nd-Ab is ignored.

This investigation provides evidence that the 2nd-Ab is a major and critical factor for reliable determination of MFIs of serum anti-HLA Abs. The following evidence shows that IgHPolyFab may be more sensitive and more reliable than IgHMon Fab for monitoring anti-HLA Abs in normal sera, in IVIg, or in allograft recipients before and after transplantation. MFIs of negative controls obtained with IgHPolyFab were significantly higher than those obtained with FcMonoIgG (Fig. 1); the number of HLA Ags that were recognized in native sera or purified IgG from sera (Table I), therapeutic IVIgs (Table IV), or transplant recipients’ sera (Table VIII) is consistently and markedly higher with IgHPolyFab than with FcMonoIgG, despite the high MFI background in the negative controls; and MFI of a number of HLA-I and II Ags was positive (>500 MFI at 1:10 dilution) for volunteer serum IgG, purified IgG (Tables II, III), and different IVIg preparations (Tables V, VI) only with IgHPolyFab (in striking contrast, negative or low MFIs were noted for many of these “IgHPolyFab” Ags” when tested with FcMonoIgG).

These findings validate our proposed hypothesis. The very concept of “sensitization” (augmented anti-HLA IgG Ab production) in patients waiting for donor organs is based on the number of serum HLA Abs identified with IgHPolyFab (provided by the vendor who supplies Luminex Multiplex SAB assays to the laboratories involved in clinical transplantation). Obviously, the number of allo-HLA Abs, which may include pre-existing DSAs and non-DSAs, determined using IgHPolyFab as the 2nd-Ab could be due to false positivity. There is no doubt that IgHPolyFab, when used as 2nd-Ab in immunohistochemistry and histopathology, is of significant benefit, because the intensity of the fluorescent signal is highly enhanced by polyclonal F(ab’2) binding to cell surface–bound Abs. Most likely, this feature is well appreciated by pathologists, and they probably would have advised the vendors supplying Luminex Multiplex SAB assays to choose IgHPolyFab. However, the use of IgHPolyFab without validation for immunoassays, particularly for the simultaneous monitoring of several Abs in a Luminex Multiplex SAB assay, will surely produce misleading assessment of Abs (false positivity) in the sera of patients waiting for donor organs. MFIs obtained with IgHPolyFab are a yardstick for clinicians to desensitize the patients by

FIGURE 4. Disparity in the serum titrimetric profile of MFIs for de novo DSAs against HLA-A*24 (A), HLA-DR52 (B), and HLA-DQ7 (C) Abs is obvious in the sera of an allograft recipient (Ren-6) tested with IgHPolyFab (closed symbols) and FcMonoIgG (open symbols). The high MFI of the Ab reveals the DSA. (D) Differences in the titration of serum anti-DR52 Ab when tested with different dilutions (concentrations) of 2nd-Abs. The original concentration of both 2nd-Abs is 0.5 mg/ml. Each 2nd-Ab was tested at 1:20, 1:80, and 1:160 dilutions. At 1:800 dilution of sera, IgHPolyFab at 1:20 dilution (▲) and FcMonoIgG at 1:160 dilution (▲) render the same MFI (>5000). IgHPolyFab recognition of the primary Ab is less efficient than that of FcMonoIgG.
administering immunosuppressive therapies; some of them are cytotoxic to all immune cells (e.g., anti-thymoglobulin Abs) (36), and others contain higher levels of HLA-I and HLA-II Abs (e.g., IVIg or blood transfusion from female donors) (29, 30). It is well known that immunosuppressed allograft recipients succumb to infection (37–43).

In almost all posttransplant patients examined in this study, a paradoxical finding with regard to MFI values emerges. MFI values obtained with FcMonoIgG are invariably higher than those obtained with IgHPolyFab or even with FcPolyIgG (Figs. 2, 3), which requires further clarification. We attribute this phenomenon to the increased density of serum Abs in general (44–46), and anti-HLA Abs in particular (47), consequent to transplantation. Transplantation induces immune events leading to the production of inflammatory mediators, and it brings about inflammation that has a tremendous impact on the immune and tissue components of the allograft, as well as on those of the recipient. Open surgery, per se, is known to result in inflammation, and it releases the proinflammatory cytokine IL-6 (48). In addition, inflamed allograft endothelial cells, smooth muscle cells, and, above all, the diverse immune cells infiltrating into the inflamed allograft elicit production of proinflammatory cytokines, such as IL-6 (48). The approximate concentration of IL-6 in the serum of a healthy individual is 1 pg/ml (10^7 molecules per milliliter), which increases >100-fold upon inflammation or injury (49). IL-6–induced alterations in the allograft microenvironment include the activation, maturation, and proliferation of naïve B cells to plasma cells, which leads to the production of high-affinity Abs (50). Therefore, inflammation is the major inducer of de novo Ab formation against MHC and non-MHC Ags, because IL-6 functions as the B cell differentiation factor and B cell stimulatory factor-2 and is involved in Ab-mediated transplant vasculopathy and loss of graft (51, 52). Therefore, inflammation could be a factor for the marked increase in serum IgG concentrations observed against alloantigens, autoantigens, and anti-idiotypic Abs before and after transplantation (44–47).

Fig. 5 illustrates the rationale that the higher MFI values obtained using FcMonoIgG compared with IgHPolyFab and FcPolyIgG for the sera of allograft recipients are due to the higher density of serum IgG Abs. The figure further illustrates that MFIs obtained from sera or IgG purified from the sera of normal individuals or from IVIg (plasma pooled and purified from tens of thousands of normal individuals) using IgHPolyFab are higher than those obtained using FcMonoIgG as a result of the lower density of serum IgG Abs. When Abs are low in density, IgH is exposed for multiple binding to the specific Fc of the anti-HLA Abs bound to Ags. In these circumstances, the 2nd-Ab devoid of the Fc region minimizes nonspecific binding of polyclonal F(ab')_2, and the fluorescent intensity is exaggerated (a possible causal factor for false positivity of HLA reactivity).

When Ab production is augmented during transplantation, the titrmetric profile of MFIs obtained with FcMonoIgG also shows greater Fc affinity. In contrast, IgHPolyFab fails to bind to IgH as a result of the increase in the density and consequent aggregation of IgGs on SABs. Persistently higher MFIs for all DSAs at the highest dilution of serum with FcMonoIgG validate the specificity, sensitivity, and, above all, the reliability of FcMonoIgG. The use of FcMonoIgG ensures a 1:1 ratio of primary Ab/2nd-Ab. In contrast, polyclonality of IgHPolyFab that binds to both of the constant regions of IgH of a single primary Ab is not accessible when the density of the primary Ab bound to Ag-coated beads is high, which would underestimate the MFI of the primary Ab; the reverse is true when serum IgG density or concentration is low (Fig. 5).

The polyclonal F(ab')_2 is indeed useful for visualization of IgG bound to cell surfaces or tissue sections, because it provides signal amplification that occurs as multiple F(ab')_2s bind to the HC of Ag-bound IgG. In addition, the size of F(ab')_2 compared with a whole IgG enables better penetration into tissues. Furthermore, the 2nd-Ab devoid of the Fc region minimizes nonspecific binding of the 2nd-Ab to Fc receptors on the cell surface. However, none of these benefits of IgHPolyFab are applicable for monitoring MFIs of anti-HLA IgG or even other autologous or allogeneic Abs when used in Luminex Multiplex SAB assays.
In summary, it may be noted that this investigation highlights the impact of the clonality (polyclonal versus monoclonal) and the nature of F(ab′) fragments versus whole IgG of the 2nd-Ab when using the highly sensitive dual-laser Luminex platform for detecting and monitoring the concentration of anti-HLA IgG. When serum IgG concentration is low, the PE-conjugated polyclonal F(ab′) fragments are bound to multiple epitopes on one primary anti-HLA IgG. When the serum IgG concentration is high, as in posttransplant sera, MFI values are underestimated, because all of the epitopes of the primary anti-HLA IgG targeted by the polyclonal F(ab′)2 fragments may be sterically hindered. In contrast, MFI values obtained with FcMonolG are not affected by the high concentration of primary anti-HLA IgG because the monoclonality ensures a 1:1 ratio of primary anti-HLA IgG/fluoresceinated monoclonal IgG, and the Fc specificity ensures epitope accessibility of the 2nd-Ab, even when the primary Ab concentration is high.

Therefore, from the clinical perspective of evaluating MFIs of anti-HLA IgG or any other autoantibodies or alloantibodies, the use of FcMonolG for monitoring pretransplant sera may increase the organ accessibility of patients on the waiting list by reducing unrealistic MFI amplifications of alloantibodies. This misleading allo-HLA reactivity of serum IgG (false positivity) will deny a donor organ for a patient, because it fails to reveal the precise level of sensitization for therapeutic evaluation.

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Disclosures
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