Introduction

"The body of man has in itself blood, phlegm, yellow bile, and black bile; these make up the nature of the body, and through these he feels pain or enjoys health. Now, he enjoys the most perfect health when these elements are duly proportioned to one another in respect to compounding, power and bulk, and when they are perfectly mingled. Pain is felt when one of these elements is in defect or excess, or is isolated in the body without being compounded with all the others."

When the Greek philosopher/physician Hippocrates first wrote these words, approximately 24,000 years ago, the practice of science and medicine was largely the domain of mystics, charlatans and magicians. The Greeks of Hippocrates time became adept at observing and codifying outward symptoms of disease (calor, rubor, tumor and dolor). The underlying cause for disease, however, was attributed to an “imbalance” in the four “humors”, nebulous ill-defined entities that were recalcitrant to systematic study. This is understandable, as it is only within the last century that the tools and technologies necessary to understand the molecular basis for disease have become available.

A prime example of the advancement in mankind’s scientific knowledge is the study of immunology (in general) and antibodies (specifically). In the late 1800s and early 1900s, the efforts of scientists such as Elie Metchnikoff, Paul Erlich, and Louis Pasteur laid the groundwork for a revolution in the way in which we view pathologic processes. The form, function and role of antibodies in human health and disease was more finely elucidated throughout the 20th century, coinciding with advances in medical and surgical technology that made human solid-organ transplantation a feasible reality. The discovery
that alloantibodies were a primary cause of allograft rejection was a watershed moment in the field of transplant medicine (1, 2).

The techniques for detecting and defining donor-specific alloantibodies (DSA) in the serum of solid-organ transplant candidates and recipients have steadily evolved in the past few decades. During the progression from standard complement-dependant cytotoxicity (CDC), to enhanced CDC (i.e. AHG-CDC), to ELISA and flow cytometric cell-based techniques, and finally to luminex or flow cytometric (FC) single-antigen bead (SAB)-based methods, there has been a consistent increase in both the sensitivity and specificity of alloantibody detection. The introduction of SAB, in particular, has allowed for the definition of individual DSA specificities even in highly sensitized patients. This, in turn, has spurred a resurgence of the concept and application of the virtual crossmatch, and potentially, a substantive revision of current organ allocation policies. However, many questions regarding the data derived from SAB technology remain: What is an appropriate cut-off value for determining positive vs. negative SAB results? Why don’t SAB results correlate to actual crossmatch results 100% of the time? What are factors that influence the ability to use SAB data to predict crossmatch results, and how can these factors be mitigated? How should SAB data be acquired and analyzed, MESF vs. MFI vs. channel shift, logarithmic vs. linear amplification? Are low-level antibodies, detectable by SAB but not FC crossmatch (XM) or CDC XM, clinically relevant?

While this article will not attempt to answer all of these questions, I will present data that relates the antigen density on the SAB to their relative sensitivity in detecting alloantibodies, and scenarios that help to explain why SAB results do not always correlate to XM results. It is my hope that readers will be able to utilize this information
to make informed decisions about how best to use and interpret SAB data in their own laboratories, for the benefit of the transplant centers and patients they serve.

**Materials/Methods**

**SAB Testing**

FlowPRA Class I and II single-antigen beads were obtained from One Lambda, Inc. (Canoga Park, CA, USA). Briefly, SAB were incubated with serum, incubated, washed, and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG. Following a final wash, the SAB were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). To quantitate the density of HLA on the Class I SAB, a FITC-conjugated monoclonal antibody against HLA-A, B and C (clone G46-2.6, BD Biosciences) was utilized. Isotype control antibody was also run to confirm absence of non-specific staining. MESF (molecules of equivalent soluble fluorochrome) values were obtained by using the Quantum FITC MESF kit (Bangs Laboratories, Inc., Fishers, IN, USA), according to manufacturers directions. All MESF values are given as Units x 10^{-3}.

**FCXM Testing**

Frozen lymphocyte suspensions were thawed and incubated with 2 mg/ml pronase (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes, washed, and 5 x 10^4 of the lymphocytes were then incubated with 20 ul serum. After 30 minutes, the cells were washed, and then stained with FITC-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA), PE-conjugated goat anti-human IgM (Caltag/Invitrogen, Carlsbad, CA, USA), PerCP-conjugated anti-human CD3 (BD
Biosciences) and APC-conjugated anti-human CD19 (BD Biosciences). After a final incubation and wash, the cells were analyzed on a FACSCalibur (BD Biosciences). MESF values were obtained as described above. In Table 2, mean channel shift (MCS) values (MFI of cells incubated with specific sera – MFI of cells incubated with negative control serum, 256-channel scale) are utilized. A T-cell MCS of >15 is considered to be positive in our laboratory.

Results

Variable HLA Density on SAB

The HLA density ranged from 10.1 MESF on the HLA-A69 SAB to 333.6 MESF on the HLA-A31 SAB. The large degree of variability in antigen density of each available HLA-A and -B SAB is shown in Figure 1. The antigen density on the beads also varies considerably on Class II SAB, from lot-to-lot, and on SAB utilized on a luminex platform (data not shown).

SAB versus FCXM

After determining the HLA density on each HLA Class I SAB, we identified archived sera that contained antibodies against specific SAB with low, medium and high density HLA (Fig. 1, arrows). Serial dilutions of these sera were tested against SAB and lymphocytes (in a FCXM) expressing the cognate antigen. Sera/cell combinations were chosen such that only a single HLA specificity was targeted in each FCXM. When using antisera against HLA targets with low density on SAB (HLA-A69 and –B13), the MESF values of the undiluted (1:1) sera in the FCXM were higher, or equivalent to the values obtained with the SAB (Figure 2 and Table 1). In addition, these antibodies were detected (i.e. positive by our laboratory’s criteria) at higher dilutions in the FCXM,
compared to SAB (Table 1). In contrast, when using sera with alloantibodies against HLA targets with medium (HLA-A23 and –AB8) or high (HLA-A31 and –A66) antigen density on SAB, the undiluted sera uniformly produced higher MESF values with the SAB, compared to the FCXM. In each case, except for the HLA-A66 antiserum in which the endpoint was the final 1:512 dilution for both assays, the endpoint dilution for antibody detection was higher with the SAB, compared to the FCXM (Table 1).

Additive Effect of Weak Antibodies

The preceding results demonstrate that antibodies may be detected with SAB that are too low in titer to cause a positive FCXM against cells expressing the cognate HLA. However, would these alloantibodies be capable of causing a positive FCXM if a serum with multiple weak antibody specificities was tested against cells expressing multiple cognate HLA? To test this, a serum exhibiting multiple weak (i.e. ≤ 1500-2000 MFI by luminex SAB) alloantibodies was tested by FCXM against lymphocytes from seven donors. The donors were chosen to express 1, 2, 3, or 4 Class I HLA cognate for these antibodies. As the antigenic target load of each cell increases, the FCXM results become increasingly more-positive (Table 2). While the results are not strictly “additive” in a formal mathematical sense, these data demonstrate that low-titer antibodies causing negative or weakly-positive FCXM are capable of causing clearly positive FCXM if cells express multiple HLA targets of these antibodies.

Discussion

The commercial availability of microspheres coupled to single-antigen proteins represents a technological and paradigmatic breakthrough in the field of clinical histocompatibility practice. The ability to define alloantibody specificities, and quantitate
the concentration of such antibodies in the serum of transplant candidates and recipients provides new tools to generate potentially useful clinical data. Two prominent examples are the ability to perform a virtual XM, even in highly sensitized patients (3), and determining the efficacy of desensitization protocols (4). The clinical relevance of data derived from this technology, and therefore the optimum utilization of the information obtained, however, is not fully understood. For example, the sensitivity of SAB in detecting alloantibodies is largely related to the antigenic density of the proteins displayed on their surface, as illustrated in the data shown in Figure 2. This has significant implications for the accuracy of virtual XM. An antibody that appears to be weak (<1500-2000 MFI) on a bead population with low-density HLA will very often result in a positive XM against cells. In contrast, a weak antibody detected on beads with high-density HLA will usually result in a negative crossmatch. Therefore, setting a threshold cut-off value for positive SAB results is not absolute. It requires knowledge of the differing protein density on the SAB population, and experience in correlating SAB sensitivity to XM sensitivity in your own laboratory.

It is relatively simple to predict a negative XM in an unsensitized patient, and predict a positive crossmatch in a patient with clearly-defined, high-titered DSA (against loci for which the donor has been typed), but predicting a negative XM in a patient with low-titered multiple-specificity alloantibodies is much more problematic (5). While it may seem attractive to be able to achieve a highly accurate rate (>90%) for predicting negative XM, this is unrealistic for highly sensitized candidates. Indeed, setting a strict criterion for assignment of unacceptable antigens (i.e. setting the threshold cut-off very low) will inevitably lead to the exclusion of donors which may in fact be XM-compatible. In the
Seattle transplant program, the centers we serve have agreed to a cut-off for unacceptable antigen assignment of approximately 2000 MFI (luminex SAB). Antibodies above this cut-off usually cause a positive FCXM. Antibodies below this cut-off are noted and tracked (assigned as “2nd tier” antibodies), and will sometimes cause positive XM, especially if the donor is homozygous for the cognate antigen. This strategy has decreased our rate of negative crossmatch prediction for highly sensitized candidates to ≤ 50%. However, it allows for transplantation of candidates that would have otherwise been denied access, because the donor would have been electronically ruled out. In a recent one-year period, 19% (3/16) of the transplants performed for >80% PRA recipients went to patients with a 2nd tier DSA, which were not entered into UNet as strictly unacceptable antigens. The short-term graft survival has been 100%, with no difference in immune-mediated complications compared to DSA-negative recipients. While the short- and long-term clinical relevance of DSA detectable by SAB, but not XM, has been the subject of several recent publications, the findings are varied, reflecting differences in patient populations, immunosuppressive protocols, and crossmatch techniques (6-10). This remains a very important question in this field.

In addition to variable antigen density on their surface, which makes correlation of SAB-sensitivity to XM-sensitivity problematic, SAB technology, and the way the data obtained from SAB testing is utilized, has several other shortcomings:

1) The HLA on the beads is not necessarily the HLA expressed by the donor (for allele-specific alloantibodies, high-resolution HLA typing of the donor may be required, or if the donor expresses an HLA not represented on the beads, an alloantibody may be missed).
2) It is possible to detect antibodies against HLA that are not routinely identified by typing (high resolution for allele-specific abs, DQA, DP, Cw, etc.). If the antibody specificities are defined, but the corresponding donor HLA type is not, half of the equation necessary for virtual crossmatching is missing. This is an example of policy lagging behind technology.

3) The manufacturing process may lead to denatured HLA on the beads, exposure of cryptic epitopes, and false reactivity that is not truly HLA-specific.

4) Some patients have a high degree of non-specific reactivity against solid-phase assays, and accurate identification of HLA alloantibody specificities can be difficult.

5) Alloantibody specificities and titers may change over time. Since it is unreasonable and unjustifiably costly to perform antibody specificity testing on a frequent basis, a patient may have an antibody now that was not previously detected (temporal changes).

6) Different transplant centers have different levels of tolerance for DSA titers. For instance, one center may rely solely on CDCXM, and another may rely on FCXM, to determine recipient-donor compatibility. In addition, different HLA labs have different levels of sensitivity for detecting alloantibodies, even if they are using the same assay (i.e. FCXM). If widespread geographic donor sharing for transplant recipients is to be implemented, predicated upon virtual crossmatching, standardization of assays and reporting of units of measurement will be necessary. Even a brief perusal of the relevant current literature will reveal broad inconsistencies in the manner in which laboratories perform assays and report results.

Despite these limitations, the single-antigen assay for determining alloantibody specificity and concentration is, in my opinion, the most important development in
histocompatibility testing for solid-organ transplantation since the prospective crossmatch. Although this technology raises many questions, and frequently provides data that we are currently ill-equipped and under-funded to manage and utilize, it represents a significant and welcome shift away from old paradigms. To anyone who has been working in the field of histocompatibility for decades, it sometimes seems like it is truly magical. We are now reaching a point where the humors may not seem to be so nebulous, after all.

References


Figure 1. HLA density on Class I HLA-A, B SAB. Each solid bar represents the mean value of independent triplicate measurements. Error bars represent 1 standard deviation from the mean value. Arrows indicate the SAB which were used to compare sensitivity of SAB vs. FCXM (Figure 2).
Figure 2. Titration curves of HLA-specific polyclonal antisera tested against SAB and cells (FCXM).
Table 1. SAB HLA Density and SAB vs. FCXM Results.

<table>
<thead>
<tr>
<th>HLA</th>
<th>SAB Density(^1) (MESF x 10(^{-3}))</th>
<th>Positive End-Point Dilution(^2)</th>
<th>Sensitivity Diff. SAB vs. FCXM</th>
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<tbody>
<tr>
<td>A69</td>
<td>Low (10.1)</td>
<td>1:1</td>
<td>- 4 x</td>
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<tr>
<td>B13</td>
<td>Low (17.4)</td>
<td>1:128</td>
<td>- 4 x</td>
</tr>
<tr>
<td>A23</td>
<td>Med. (66.1)</td>
<td>1:64</td>
<td>+ 4 x</td>
</tr>
<tr>
<td>B8</td>
<td>Med. (78.5)</td>
<td>1:64</td>
<td>+ 4 x</td>
</tr>
<tr>
<td>A31</td>
<td>High (333.6)</td>
<td>1:512</td>
<td>+ 8 x</td>
</tr>
<tr>
<td>A66</td>
<td>High (292.6)</td>
<td>1:512</td>
<td>ND*</td>
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1. Data from Figure 1
2. Data from Figure 2

*Not Determined. Positive end-point not reached.
Table 2. Additive effect of multiple weak antibodies against cells in a FC XM assay. A single antiserum with weak antibodies against A2, A11, B55 and B60 was tested by FCXM against cells expressing cognate HLA (left column, bold underlined). The MCS of the diluted serum in the FCXM is shown to the right. A MCS $\geq 15$ is considered positive (bold, underlined).

<table>
<thead>
<tr>
<th>HLA type of cells (Class I)</th>
<th>Serum Dilution</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
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<td>A1, A24, B8, <strong>B60</strong></td>
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<tr>
<td>A1, <strong>A11</strong>, B35, B60</td>
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</tr>
<tr>
<td><strong>A2</strong>, <strong>A11</strong>, B35, B60</td>
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<tr>
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