Chapter 17

HLA Antibody Detection and Characterization by Solid Phase Immunoassays: Methods and Pitfalls

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Abstract

Solid phase immunoassays for the detection and characterization of HLA-specific antibodies provide greatly increased sensitivity, specificity, and time and reagent efficiency, compared to the traditionally used cell-based methods. Testing is performed using commercially available test kits. The assays are of two general types: enzyme-linked immunosorbent assays and multianalyte bead. The types vary in both sensitivity and equipment requirements.

While these assays afford great improvement over the cell-based assays, they can be confounded by interference from substances within the serum that result in high background reactivity. The high sensitivity of the assays also makes them more susceptible to environmental factors and operator variability. The user must be aware of the capabilities of the various formats, the factors that can affect test results, and lot to lot variability of any single product. Knowledge of the characteristics of each product and thorough and accurate analysis of the results are essential to the utility of these assays.

Key words: Enzyme-linked immunosorbent assays, HLA antibody, HLA phenotype panel, Luminex®, Multianalyte bead assays, Pooled antigen panel, Single antigen panel, Solid phase immunoassays

1. Introduction

For more than 30 years, detection and characterization of HLA-specific antibodies (HLA-Ab) were performed using cell-based methods. These methods were hampered by the requirement for a sufficient quantity of viable lymphocytes, low specificity, and prior to the use of flow cytometry, low sensitivity. Isolation of soluble HLA antigens was achieved in the mid 1970s (1), but it was 20 more years before there was success in maintaining the native conformation of soluble HLA molecules following fixation to a solid phase (2, 3). The development of solid phase immunoassays (SPI)

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for HLA-Ab provided enormous advantages for both HLA-Ab testing for transplantation and the investigation of serologically defined HLA epitopes. Testing by SPI reduces reactivity of antibodies to non-HLA antigens, thereby increasing specificity, increases sensitivity significantly, provides evaluation of antibody strength on a continuous scale facilitating manual analysis and determination of specificity, and can be partially or fully automated. Further advantages are that SPI are available as kits from several manufacturers, allowing laboratories to avoid the laborious task of preparing tests and providing some measure of consistency among laboratories. However, optimizing utility of these assays requires thorough and ongoing quality control. As of this writing, kits are available for the characterization of antigens encoded by antigens of the HLA-A, -B, -C, -DRB1, -DRB3-5, -DQA, -DQB, -DPA, -DPB, and MICA loci. Collectively, these kits test for antibodies to most, but not all, HLA antigens which would be impossible given the polymorphism of the HLA system. Additionally, many of the kits provide the allele identity of the target molecule.

This chapter will discuss the types of assays commercially available, the test procedures, manufacturer-provided materials and reagents and those that must be provided by the laboratory, advantages and disadvantages of the various tests available, test capabilities, problems with and shortcomings of the various tests, and clinical applications. Manufacturers provide detailed instructions for performance of their tests, and therefore, descriptions of the assays provided here will be somewhat generic. Specific information not included here can be found at the web sites of the various manufacturers which are listed below in alphabetical order. Note: since manufacturers may introduce new products or platforms or modify existing ones, web sites should be checked for such changes before deciding to use a particular system.

GEN-PROBE: http://www.gen-probe.com/products-services/
ONE LAMBDA: http://www.onelambda.com

1.1. Commercially Available Assays

1.1.1. Formats

Kits for the detection and characterization of HLA-Ab are available in three formats: pooled HLA antigens, panels of individual HLA phenotypes, and panels of single HLA antigens (4, 5). Pooled antigen kits, as the name implies, consist of a pool or mixture of various HLA antigens and generally include the most common HLA specificities found in several racial groups. The pools may be derived through isolation of antigens from the cells of hundreds of individuals or by mixing antigens prepared in transfected cell lines or baculovirus systems. The pools may be comprised of class I (cI) antigens only, class II (cII) only, or a mixture of cI and cII if the platform used can distinguish the individual antigen classes in a mixture. Phenotype panels are comprised of an array of individual

cI or cII phenotypes while single antigen panels have the HLA antigens present as individual, purified antigens.

The pooled antigen test provides a rapid and cost-efficient system for determining the presence or absence of HLA-Ab. This test does not determine specificity. However, it does provide an indication of relative strength and breadth of antibody making it useful when monitoring sequential sera from a patient (4). Phenotype panels provide an opportunity to determine antibody specificity and strength. These panels frequently include a phenotype that includes two or more antigens of a potential donor, enhancing the ability to assess the collective strength of the antibodies against that donor. Further, each antigen is almost always present in multiple phenotypes providing a safeguard if one phenotype fails to react properly. Sera reactive with more than 95% of the panel may make it difficult to determine, conclusively, the presence of an antibody to antigens represented by a limited number of phenotypes. However, because the phenotypes that all bear the target of an antibody cluster together when the reactions are listed in descending order of strength, many antibody specificities can be identified in very broadly reactive sera. Single antigen panels permit identification of antibodies that may be masked by other antibodies or react poorly in phenotype panels. Further, since these panels often include multiple alleles within an antigen group, it is easier to detect antibodies reactive with only some alleles within an antigen group. Although single antigen panels have the ability to detect most, if not all, HLA-Ab present in a serum, there are several things to be considered. It is difficult to assess the collective strength of multiple antibodies in a serum as it has been shown that the collective strength of the antibodies is not equivalent to the sum of the strengths of reactivities with the individual antigens (6). Further, the failure of one antigen may not be recognized which may result in the failure to detect antibody to that antigen. As expected, the overall sensitivities of the assays increase with the amount of individual antigen on each bead, so that the order of sensitivity is single antigens > phenoytpes > pooled antigens.

1.1.2. Assay Types, Matrices, and Platforms

Two general types of assays are available, multiplex or multianalyte bead assays and enzyme-linked immunosorbent assays (ELISA) (4, 5, 7). For both types of assay, commercial kits provide the matrix with bound soluble HLA antigens. The bead assays are performed on either a conventional flow cytometer or a Luminex® fluoroanalyzer, depending on the kit. In these assays, polystyrene beads are impregnated with fluorescent dyes and beads bearing different pools of antigens, phenotypes, or single antigens are differentiated by the fluorescence of the bead. After incubation with control or test sera, a fluorochrome-labeled anti-human globulin is added to detect beads that have antibody bound. When tested in a conventional flow cytometer, beads of different fluorescence are identified on the

FL2 channel while the intensity of fluorescence is read on the FL1 channel. Multiplex beads for use with the Luminex® platform are incorporated with different combinations of ten dilutions of each of two different dyes to yield 100 different beads. The Luminex® fluoroanalyzer has two lasers: one of which identifies the bead and the other identifies beads with bound antiglobulin. In both assays, different levels of reactivity can be used to approximate relative antibody strength. Depending on the assay format and manufacturer, these assays utilize 10–20 μL of test serum per test.

ELISA are performed on either microtiter or microtest plastic plates. The procedure is comparable to that for the bead assays except that the antiglobulin is bound to an enzyme and test reactions are detected colorimetrically after addition of a color-producing substrate. The required serum volume is approximately 300 μL. In general and depending on the cutoff value used for positivity, the bead-based assays are more sensitive than are the ELISA (8–10).

The antiglobulin reagent provided with all assays is specific for human IgG. It is possible to use reagents specific for other classes of immunoglobulin and for IgG subclasses (11); however, the titer and specificity of those reagents must be verified by the user. Assays have also been developed for the detection of the C4d and C1q components of complement (12–14). However, in our experience, the values obtained for IgG subclasses and C4d may be low (15) and may be more applicable to determining relative quantities.

2. Materials

2.1. Bead-Based Assays

Materials Provided (see Note 1)

1. Analysis software.
2. Beads with bound HLA antigens (see Note 2).
3. Fluorochrome-labeled antiglobulin (varies among manufacturers).
4. Negative control serum (varies among manufacturers).
5. Positive control serum (varies among manufacturers).
6. Wash buffer.

Materials Required But Not Provided

1. Adhesive plate sealers.
2. Instrument: flow cytometer or Luminex® fluoroanalyzer, depending on kit.
3. Sheath fluid.

4. Calibration beads.
5. Microcentrifuge.
6. Microfuge tubes.
7. Millipore filter plates or microtiter plates.
8. Multiscreen vacuum manifold (kit dependent).
9. Plate holder for centrifuge.
10. Rotator or plate shaker.
11. Various adjustable and multichannel pipettes.
12. Vortex mixer.
13. Computer.

2.2. Conventional ELISA

Materials Provided

1. Microtest plates or well strips for use in microtiter plates, preloaded with HLA molecules (see Note 3).
2. Enzyme-conjugated anti-human IgG.
3. Antibody diluent.
4. Colorimetric enzyme substrate (varies among manufacturers, see Note 4).
5. Substrate buffer (varies among manufacturers).
6. Control sera (varies among manufacturers).
7. Plate sealers.
8. Stop reagent.
9. Wash buffer.

Materials Required But Not Provided

1. 37°C water bath or incubator.
2. Deionized water.
3. Tubes for sample and reagent dilution.
4. Microplate washer (optional).
5. Adjustable pipetors and Multichannel pipetor with tips.
6. Plate reader with appropriate filters.†

† Adapted for microtest plates when necessary.

3. Methods

3.1. Sample Preparation (See Notes 5–7)

1. Obtain blood samples in tubes without anticoagulant.
2. Separate and centrifuge serum to remove aggregates. Do not use sera that are lipemic, hemolyzed, microbially contaminated, or heat-inactivated.

3. Store sera at 4–8°C for up to 48 h or below 35°C for longer periods. Serum to be stored longer at 4–8°C should have sodium azide added to a final concentration of 0.1% to prevent microbial growth.

3.2. Bead-Based Assays

Generic Procedure (see Note 1).

1. Reagents such as wash solution and PE-IgG must be thoroughly mixed prior to use.
2. Pre-wet wells (when using filter plates on the Luminex platform only).
3. Add serum and beads. Manufacturers specify volumes of each. Bead suspensions should be mixed well periodically to assure even dispersion of the beads (see Note 2).
4. Incubate 30 min on a shaking platform to allow specific binding of antibody, when present, to bead-bound antigen. Shaking is necessary to prevent beads from settling which will reduce contact with antibody (see Note 8).
5. Discard supernatant fluid (see Note 9).
6. Wash beads (number of washes is manufacturer specific) to remove unbound immunoglobulin (see Note 10).
7. Discard supernatant fluid and add labeled antiglobulin reagent.
8. Incubate 30 min on a shaking platform.
9. Wash to remove unbound antiglobulin and resuspend beads (see Note 10).
10. For delayed acquisition with a flow cytometer, add a fixing solution.
11. Acquire beads (see Notes 11–17).
12. Perform analysis using manufacturer-provided software. Total time needed for procedure is approximately 4 h excluding data review and analysis. Times will vary depending on the use of robotic fluid handling systems, operator experience, and number of samples tested.
13. In addition to control sera, bead sets have built in control beads.

3.3. Conventional ELISA

Generic Procedure (see Note 3)

1. Dilute all reagents and sera according to manufacturer's instructions. Include a negative serum with each run to establish background reactivity.
2. Pre-wet test wells, let stand, aspirate fluid. This will rehydrate the bound antigens.
3. Add pre-diluted test and control serum to wells of microtest trays or microtiter strips (see Note 4).

4. Cover tray and incubate 30–45 min at 37°C (GenProbe) or 1 h at 20–25°C (One Lambda) (see Note 18).
5. Remove liquid from wells and wash three times to remove any unbound immunoglobulin (see Note 19).
6. Add conjugated antiglobulin reagent.
7. Cover tray and incubate 30–45 min at 37° (GenProbe) or 40 min at 20–25°C (One Lambda).
8. Remove liquid from wells and wash to remove unbound antiglobulin (see Note 20).
9. Add enzyme substrate (see Note 21).
10. Incubate in the dark 30 min at 20–25°C (GenProbe) or 10–15 min at 37°C (One Lambda) to allow the reaction to equilibrate (see Note 22).
11. Add stop reagent in the same sequence as for substrate. Trays may be stored in the dark for 30 (GenProbe) or 60 min (One Lambda) (see Note 23).
12. Read absorbance (optical density) at 405–410 nm (GenProbe) or 630 nm (One Lambda) (see Note 24).
13. Perform analysis using manufacturer-provided software or manually utilizing recording sheets provided with kit. The percent PRA is calculated by dividing the number of positive HLA-Ab-containing wells by the total number of antigen preparations in the panel. The cutoff value for positivity is calculated as a percentage of the range of reactivity of the provided serum control (SC) tested in the positive wells minus the non-specific background of the test serum (or antibody diluent) tested in the blank wells (One Lambda) or two times the OD value of the mean of the negative control wells (GenProbe). The assignment of antibody specificity is aided by ranking the reactions with test sera in descending order of strength.

Total time needed for procedure is approximately 4 h excluding data review and analysis. Times will vary depending on the use of robotic fluid handling systems, operator experience, and number of samples tested.

4. Notes

1. GenProbe reagents are provided together in a kit and are lot-specific. One Lambda reagents are sold individually. GenProbe pooled antigen and phenotype panels must be stored at 4°C. GenProbe single antigen beads must be stored at −80°C and can be refrozen up to six times. One Lambda single antigen,

pooled antigen, and phenotype beads must be stored at –65°C. They can be frozen only once, but they may be stored at 4°C up to 3 months.

2. Beads should be kept in suspension by periodic mixing on a Vortex mixer while being added to tubes or wells.
3. Reagents and trays should be stored according to manufacturer's instructions. Trays and microwell strips/plates should be protected from moisture.
4. Be careful not to touch the well bottom. Change pipet tips regularly to avoid cross contamination.
5. Substances inherent in the serum or external agents such as therapeutic antibodies may interfere with test results. We have shown that low values for the internal positive control (approximately <8,500 MFI for single antigen panels and <12,000 MFI for phenotype panels) or high values for the internal negative control (approximately >150 MFI for all negative controls or >500 MFI for any one control for panels with multiple negative controls, or >200 MFI for panels with a single negative control) indicate test interference that may result in reduced values with HLA targets and that may alter the specificities detected (16). Substances inherent in the serum may include high levels of IgM, immune complexes, and antibody to plastic, among others. There are two commercial bead-bound products for the reduction of background in Luminex® assays: SeraClean™ from GenProbe and Adsorb Out™ from One Lambda, Inc. For both products, the beads are added to a serum known to have high background reactivity, incubated, and then removed, ideally with whatever is causing high background bound to the beads. The results are variable and are probably affected by the amount and nature of interfering factors. Also, it is not possible to know if this process also reduces or dilutes HLA-Ab. We have shown that hypotonic dialysis, dialyzing serum against distilled water, is effective in eliminating or substantially reducing high background in nearly all cases. This procedure is based on the differing solubilities of IgM and IgG in distilled water which results in the precipitation of IgM but not IgG. It is possible that this procedure also eliminates some immune complexes. Dithiothreitol (DTT), a reducing agent, has been reported to reduce background in sera tested on single antigen beads (17), but in our experience it actually increases reactivity with the negative control and is not as effective as hypotonic dialysis in restoring normal reactivity to test sera in the Luminex® assay. Interestingly, we have found that sera tested on glass microchips often do not display the high background that they exhibit with a plastic matrix. Low positive and/or high negative controls indicate that reactivity with antigen-bearing beads

has been compromised and accurate interpretation of the results requires some treatment of the serum.

6. Therapeutic agents may also result in test interference. Agents shown to impact test results include Thymoglobulin, high dose IVIg, Eculizumab, and Bortezomib (18, 19). Thymoglobulin is a polyclonal rabbit serum and can thus be removed by absorbing the serum with beads coated with an anti-rabbit immunoglobulin. We have shown that hypotonic dialysis also resolves some or all of the interference caused by Eculizumab and Bortezomib.
7. Groups of patients, defined by the type of transplant, may demonstrate, on average, different levels of background or test interference.
8. All incubations should be performed in the dark at 20–25°C. Beads and fluorochrome-labeled antiglobulin are light sensitive and extended exposure may cause photo-bleaching. These reagents should be protected from light as much as possible.
9. When using filter plates, the vacuum pressure should be no greater than that required to aspirate samples. If using a vacuum manifold, do not exceed 100 mmHg. High vacuum pressure may cause beads to be crushed (misshaped) resulting in bead failures.
10. Insufficient washing may produce false negative reactions due to blocking of the antiglobulin by residual immunoglobulin in the wells. Inconsistent washing may yield inconsistent test bead and control reactivity.
11. Instruments for bead analysis should be calibrated daily or before each run if daily runs are not performed.
12. Bead acquisition should occur within the manufacturer's specified time frame.
13. Unusually high acquisition times (>20 s/sample) may be indicative of addition of incorrect bead volumes (concentrations). This may result in false negative or weak test bead reactivity.
14. If using a filter plate and one or more test wells appear clogged, scratch the plastic dimple underneath test well.
15. Low bead counts may be a result of instrument clogs and/or instrument out of calibrated range. Possible solutions are to sonicate the sample probe and/or re-calibrate instrument.
16. Inclusion of one or more well-characterized sera in a test run can provide an indication of when test sensitivity is unacceptable.
17. There is no serum control for Luminex assays; however, high positive control bead values (>20,000 MFI) and low negative

control bead values (<50 MFI), with unusually low test bead values (<100 MFI) (together) may indicate that serum was not added to the assay.

18. Test plates must be covered during incubations to prevent evaporation.
19. Inadequate washing before addition of antiglobulin will result in reduced specific reactivity due to blocking of the antiglobulin reagent by residual immunoglobulin.
20. Inadequate washing before addition of substrate will increase non-specific reactivity due to reactivity between free conjugated antiglobulin and substrate.
21. Colorimetric enzyme substrate and substrate buffer are light sensitive and should be mixed immediately prior to use.
22. Time and temperature of the substrate incubation are critical and failure to adhere to manufacturer's specifications may lead to increased background.
23. Optimal reactivity requires that stopping solution be added at the required time.
24. Plate readers should be calibrated according to manufacturer's recommendations or whenever problems arise. If trays are stored for rereading, OD values will diminish over time.

5. Data Analysis and Interpretation

Analysis software is available for all products and the manufacturers also provide calculations for performing analyses manually. The initial calculations performed by the software are to determine positivity and negativity. These calculations take into account the overall reactivity of the assay as determined from the negative control bead(s) and background noise due to individual bead variability. The values for background noise are lot specific. Also, different assays within and between manufacturers may yield different negative and positive control bead values. Thresholds for positivity can be altered by the user. The software can also perform analyses to determine specificity, correlation coefficients, chi-squared values, two-by-two analysis data for each specificity, and values normalized for antigen density. The analysis software can: provide customized reports designed by the user and lot-specific updates for background noise; allow the user to view all data for a particular patient; search by sample, patient, or test run; and generate single patient or batch reports in a variety of document formats.

Specificities may be analyzed by antigen, allele, or cross-reactive antigen groups.

We have found that computer-generated interpretation of results is not sufficient for accurate specificity assignment and that all results should be examined manually. Attention must be paid to controls. Very low negative controls may result in low test values being scored by computer analysis as positive. Low positive controls (MFI < 12,000) or high negative controls (MFI > 400) may indicate the presence of interfering factors that may result in reduced test serum values and incorrect specificity determination (this will be discussed further in Subheading 7).

When using a phenotype panel and listing reactions in descending order of strength, phenotypes bearing a common target antigen will be clustered together when the test serum contains antibody to that antigen. This can provide two types of information. First, the strongest reacting phenotypes often contain two or more antigens that are targets of antibodies in the serum, providing an estimate of the collective strength of those antibodies. Second, the test scores of these clustered phenotypes may be low and interpreted as negative by the computer but the non-random assortment of antigens strongly suggests the presence of low-level antibody.

Certain beads or wells may give consistently high results with negative control serum or consistently low or negative results with specific antibody. This information should be taken into account when assigning specificity.

Both the alpha and beta chain of HLA-DQ and HLA-DP molecules are polymorphic and interpretation of positive reactions with DQ or DP molecules should take into account the possibility of specificity to either chain. It may be possible to identify antibody directed to antigens on the alpha chain by the clustering of antigens with a common alpha chain. Greater assurance of that specificity is provided by a positive reaction with a molecule comprised of the suspect alpha chain and a beta chain found in the serum donor's own phenotype. Additionally, a serologic epitope may depend on the combination of certain alpha and beta chains (20). Specificity for this type of epitope may be difficult to define with certainty if the panel contains only one representation of that alpha-beta combination. Further confirmation may be obtained by performing a crossmatch with cells bearing the target molecule. However, this would require that the antibody is strong enough to yield a positive reaction and that the serum does not contain antibodies to other antigens on the target phenotype.

There is a maximum amount of antibody that can bind to targets. For sera that contain more antibody than can be bound to the target, the relative strength of the antibody cannot be assessed accurately. In such cases, tests of diluted serum may yield better information about antibody strength, but the user is advised to note any manufacturer's cautions or recommendations about interpreting data from serum diluted beyond the manufacturer's recommendations and to interpret data from such dilutions with caution.

6. Quality Control

Each lot of kits should be tested with human serum from a healthy, non-sensitized male or a pool of sera from such individuals. The serum should be known to lack HLA-Ab and should give unquestionable negative reactions with all or nearly all targets, individual beads, or wells (except the internal positive control if one is included in the kit). Higher than expected reactions with any target may indicate the likelihood of non-specific reactivity and/or higher than expected reaction strength with that target in the presence of antibody specific for the target. Reactivity with the negative control serum also provides information about the different background reactivities of the targets, although this should be accounted for by the manufacturer's software.

Each new lot of kits should be tested with several sera containing different, well-characterized, HLA-Ab to check the sensitivity and specificity of the lot.

Each run should include positive and negative control sera to determine the validity of the run.

Ongoing monitoring of each target should be performed to assess the possibility of inappropriately low reaction strength or increased background. The data should also be used to evaluate lot-to-lot differences in reaction strength.

Positive and negative control targets should be monitored for consistency and level of reactivity.

Whenever new targets are introduced into a new lot, their reactivity should be evaluated with antibody specific for the HLA antigen(s) of that target.

7. Technical Issues

Variability exists among formats, among platforms, among similar products from different manufacturers, between lots of the same product from the same manufacturer, between tests run on different days, and between tests run by different technologists. We have already noted that specificity and sensitivity are greater for single antigen panels than for phenotype panels and for phenotype panels than for pooled antigen panels. Not surprisingly, single antigen panels appear to be less robust than do phenotype panels. This may be due in part to the higher amounts of individual antigen in the single antigen panels. If one antigen in a phenotype is susceptible to losing its native conformation, the impact on reaction strength may be buffered, to some extent by other antigens in the phenotype. However, the effect will be more obvious when the affected

antigen is the only target. Further, the increased sensitivity of the single antigen panel may make it more susceptible to environmental variables. In phenotype panels, the effect of distortion of one antigen is greater when that antigen is in the homozygous state or when the amount of that antigen has been increased above normal by the manufacturer.

As noted earlier, we and others have observed differences in sensitivity and in susceptibility to background among the different platforms.

The single antigen products from different manufacturers differ both in the panel composition and in the sensitivity and specificity of the reactions with different antigens; therefore, use of more than one product can provide complementary information. While use of more than one manufacturer's kit may be cost prohibitive, the cost may be offset by the improved information obtained from using more than one kit, particularly when accurate assessment of antibody strength is critical.

We and others have observed significant differences in the sensitivity of different lots of the same product. Recently, we documented mean increases in sensitivity between consecutive lots of a single antigen multiplex bead kit of 5,500 MFI for the A locus and 2,400 MFI for the B locus, representing a 125–275% increase in reactivity *(unpublished data)*. On the one hand, this increased sensitivity could be beneficial if the specificity is high; but on the other hand, this is a serious problem when monitoring sequential samples from a single patient. The problem is exacerbated if the laboratory is unaware of this change until it becomes apparent after extended use of the product.

Day-to-day and tech-to-tech variability is inevitable for serologic assays but can lead to incorrect interpretation of antibody strength. After introducing a robotic liquid handling system into the laboratory, we performed an analysis of the variability between sequential duplicate tests with the pooled antigen format performed by a single technologist compared to duplicate tests performed using the robotic device. We tested sera with different strengths of reactivity. The variability in reaction strength for the technologist-performed assays was 7.9–22.8 compared to only 4.9–6.6% for the robotic-assisted liquid handling assays (21). Run-to-run variability must be accounted for when interpreting results. An example is given in Fig. 1, which shows the strength of HLA-Abs from sequential serum specimens from the same patient plotted against the strength of the positive control. The left side of the plot shows an unmistakable increase in HLA-Abs with a much more limited change in the reaction strength of the positive control. However, beginning with week 6, the HLA-Ab strength and that of the positive control are parallel suggesting that apparent changes in the HLA-Ab strength most likely reflect day-to-day differences in test sensitivity. Correct interpretation of changes in

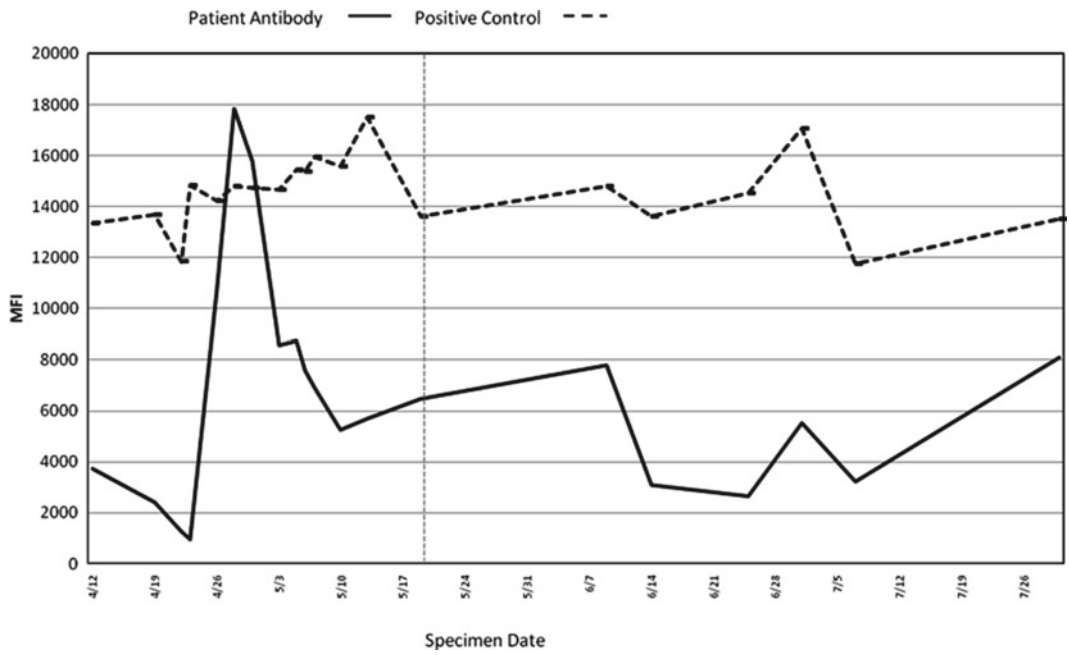

Fig. 1. Test serum vs. positive control. Run-to-run variability must be accounted for when interpreting results of HLA antibody testing. The strength of HLA-Abs from sequential serum specimens from the same patient is plotted against the strength of the positive control. The *left* side of the plot shows an unmistakable increase in HLA-Abs with a much more limited change in the reaction strength of the positive control. However, beginning with week 6 (*dotted vertical line*), the HLA-Ab strength and that of the positive control are parallel suggesting that apparent changes in the HLA-Ab strength most likely reflect day-to-day differences in test sensitivity.

HLA-Ab is critical when these data are used to determine clinical treatment.

The amount and condition of antigen is known to vary among the different HLA antigens (22). Distortion of the HLA target will yield a false positive reaction with antibody reactive with a non-native epitope resulting from the distortion of the molecule and will yield reactions that are incorrectly negative or weaker than expected with antibody to a native HLA epitope that is lost.

Differing amounts of antigen will yield different reaction strengths with a fixed amount of antibody. The amounts of Cw, DQ, and possibly DP antigens are known to be increased above that of other antigens in some kits (6). This enhances the ability to detect antibodies to these antigens, but the reaction strength cannot be considered comparable to that for other antigens, given the same amounts of antibody.

If the target bead or well is not completely coated with antigen or a blocking agent, immune complexes and immunoglobulin can adhere nonspecifically to the matrix or to the capture antibody if a capture antibody is used. This could lead to a false positive reaction. While one might anticipate that the reaction strength would be low, this may not be the only source of non-specific binding.

8. Applications

The development and use of SPI for HLA-Ab has provided a significant benefit for transplantation and for further identification of serologic epitopes. The more specific characterization of HLA-Ab has enhanced their detection. Applied prior to transplant, this information permits the identification of antibodies that are likely to yield crossmatch tests positive at different sensitivity levels such as cytotoxicity and flow cytometry. In turn, this permits calculation of the percentage of the donor population with which a patient will have a positive crossmatch (23). In the United States, this is utilized by the United Network for Organ Sharing (UNOS), the administrator of the National Organ Procurement and Transplantation Network. Using allele and haplotype frequencies for HLA-A, -B, -DRB1, and -DQB antigens from thousands of donors from four racial groups, UNOS has established a program for calculating this percentage, referred to as the calculated PRA (CPRA). An online program is maintained by UNOS and can be accessed at http://optn.transplant.hrsa.gov/resources/professionalResources.asp?index=78. Transplant programs are required to test patient sera using at least one SPI and enter unacceptable antigens from which the patient's CPRA is determined and incorporated into the allocation algorithm. Use of the CPRA has decreased the incidence of failure to transplant the intended recipient because of a positive crossmatch by 83% (24). Even without the elegant CPRA calculations, this would not have been possible without the more precise recognition of HLA-Ab made possible by SPI.

The assessment of HLA-Ab strength and specificity by SPI can also be used to perform a virtual crossmatch which has multiple applications (6, 25–31). Prior to transplantation, the virtual crossmatch based on SPI results permits procurement of organs with minimal permissible cold ischemia times, such as hearts and lungs, from a broader geographic area than does using data from cytotoxicity tests. The virtual crossmatch can expedite the process of paired donation among multiple transplant centers by eliminating the need for testing geographically distant donors who would have a positive crossmatch. The virtual crossmatch is also useful for monitoring patients following transplantation when cell-based crossmatch tests are not possible because of the presence of humanized or chimeric lymphocyte reactive therapeutic antibodies (4).

SPI are invaluable to desensitization programs. Reinsmoen et al. (32) have correlated the results of crossmatch tests with those of SPI and determined a maximum antibody strength that permits successful transplantation in patients being desensitized with high-dose IVIg. In our program, the course of donor-specific antibody (DSA), assessed by SPI prior to transplant is used to measure treatment efficacy and, in turn, adjust the treatment when indicated (33).

Post-transplant monitoring is essential since desensitized patients are at higher risk for antibody-mediated rejection (AMR) and SPI allows for rapid detection of changes in DSA strength and of the development of new DSA.

As has been shown by several groups, post-transplant monitoring of DSA, particularly in patients at higher risk for AMR, provides an opportunity for early intervention when DSA appears or increases in strength (34–40). SPI tests are more rapid and more sensitive and specific than are cell-based assays. With cell-based assays, the presence of DSA was often not detected until nephrectomy of a failed graft. This may have been due to adsorption of DSA onto the graft, reducing circulating levels of DSA below that detectable in the cell-based assays. However, SPI can detect very low levels of circulating DSA in the presence of an allograft. We have shown (41, 42) that DSA is always detected when renal biopsies are positive for C4d but that not all patients with circulating DSA have C4d positive biopsies. Thus, early detection of DSA may be a surrogate for the more invasive biopsy. We have also shown that pro-inflammatory events can cause increases in the breadth and/or strength of HLA-Ab (43). Again, early detection of these changes can be clinically relevant to patients awaiting deceased donor transplantation and to patients already transplanted.

An unresolved issue is the level of DSA that is clinically relevant. It has been shown that increased episodes of AMR are associated with DSA levels detectable by ELISA but not with levels detectable only by bead-based assays (9, 44). However, the risk incurred with DSA present only at levels detected by the most sensitive assays is one of current debate, with numerous reports both supporting (27, 45, 46) and questioning (26, 47–49) a negative impact associated with low-level antibodies. Since any amount of DSA indicates sensitization, there is a risk of a clinically relevant increase in the DSA level; therefore, monitoring of such patients is appropriate. The frequency of DSA among transplanted patients was not discernible in the era of cell-based assays. Terasaki has shown that dysfunction of renal grafts may not occur for months after the development of DSA (50). Thus, the clinical conundrum is when to intervene. SPI assessment of DSA is providing valuable information about the effects of antibodies of different strengths and different specificities and is likely to yield information that will lead to improved graft outcomes.

SPI tests of HLA-Ab combined with determination of HLA alleles have provided information about epitopes shared among alleles and have provided the opportunity to identify antibodies to an antigen that appears to be identical in a donor-recipient pair, but which is encoded by different alleles in the donor and recipient (51–53). Using SPI one can also identify antibodies to the alpha chains of DQ and DP antigens. This information is useful to determining the epitope composition of different HLA antigens and to reconcile crossmatch results that would otherwise appear anomalous (20).

9. Considerations and Recommendations

The review of the capabilities and shortcomings of various formats and platforms suggests that complete and accurate information about a patient's HLA-Abs is best obtained with the use of more than one assay. While this may appear to be cost prohibitive, by customizing the use of multiple assays according to the antibodies of a patient, we have been able to maintain test costs in the lowest quartile of independent US laboratories.

There has been much recent discussion about two issues surrounding SPI: results reporting and standardization. There is interest in having some quantitative measure of sensitization using test values expressed as MFIs or OD ratios. As we have noted above, the values are affected by several factors, including: the antibody specificity, with antibodies to enhanced antigens yielding higher values; by the format used, with higher values obtained with single antigen panels than with phenotype panels; by the sensitivity of the particular lot of reagents used; by the presence of interference causing high background and reduced test values; and by the day-to-day variability inherent in serologic assays. All of these factors must be taken into consideration for a meaningful interpretation of the test results. The trend of DSA and the crossmatch reactivity that would be predicted from the SPI results are likely to be more meaningful clinically. However, this implies that thorough and accurate analysis of the test results by the laboratory scientist and technologist is absolutely necessary to providing such meaningful interpretation.

Standardization of test results would be extremely useful for interpreting data from different laboratories. However, there are several hurdles to overcome to achieve this goal. Most importantly, at present there are no reference sera with known amounts of antibody to use as standards. Monoclonal antibodies are not representative of the polyclonal nature of antibodies present in most patients and even mixtures of such antibodies would not replicate the nature of patient's sera which contains other factors that could affect test results. Second, while some laboratories treat sera to remove interfering factors, at present this is not done universally. Third, the high degree of sensitivity of SPI and its susceptibility to variability suggests that standardization might only be achieved by considering ranges of test values. Fourth, standardization cannot be readily achieved until manufacturer-provided kits are standardized from lot to lot. The question is, how much of a problem is this. A perusal of the literature reveals that multiple variations of the cytotoxicity crossmatch have been used. Differences in incubation times and temperatures, the use of an antiglobulin, the source and specificity of the antiglobulin, and

the ratio of cells to serum can have a significant impact on test sensitivity. These same factors will affect the results of flow cytometric crossmatch tests. Further, with flow cytometric testing, there has been no standardization of the cytometers used, the antiglobulin reagent used, the reading scale, or the threshold for positivity. The only data normalization has been with the use of ratios or molecules of equivalent soluble fluorochromes. In fact, there are many publications of the results of allogeneic crossmatch results without results of autologous crossmatches which, when positive, would affect the interpretation of the allogeneic crossmatch. To date, this has not been raised publicly as an issue as has standardization of SPI. We believe that the more important practice is to have results standardized and correlated with clinical outcomes within a transplant program. However, this does not eliminate the need for appropriate testing and accurate test interpretation, nor does it preclude longer term efforts to achieve some standardization in test reporting by inclusion of criteria for the definition of clinically relevant antibodies. Despite the above noted issues, the development and use of SPI for testing HLA-Abs has had a very significant impact that has improved the care of transplant patients, our knowledge of the immunogenicity of HLA antigens, and is likely to lead to increased understanding of the humoral response to HLA antigens.

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