TRANSPLANT HISTOCOMPATIBILITY CONFERENCE FOLLOW-UP MEETING

ISSUES AND APPROACHES TO CONSISTENCY IN HLA ANTIBODY TESTING

At the conclusion of the Transplant Histocompatibility Conference (THC), jointly sponsored by ASHI, ASTS, AST, UNOS, ISHLT and NIH and held in Chicago, March 3-4, 2008, it was clear to the HLA laboratory professionals and transplant physicians and surgeons in attendance that while testing for donor specific antibodies (DSAs) has come a long way, there are still issues that need to be addressed.. Solid phase immunoassays for detecting and identifying HLA antibodies were widely recognized as a significantly more sensitive method for measuring the degree of sensitization in prospective transplant recipients than cell based methods. There was general agreement that that our goal for the use of these more sensitive antibody tests should be the ability to use the relative strength of DSAs to predict the crossmatch results and ultimately to predict the risk of graft loss. At the same time, several major areas of concern emerged regarding the use of solid phase HLA antibody test results for assessing risk in organ transplants:

- Variability between vendors, between lots, between laboratories, between technologists and between runs causing difficulty when comparing results; the need for some level of standardization or normalization of results

- Differences in effect of antibodies on different types of organ transplants and the need for organ specific risk assessment

- Clinical Correlation between test results and transplant outcomes, especially with weaker donor specific antibodies

The THC ended with a recommendation to conduct further studies to answer these concerns and to convene a second Transplant Histocompatibility Conference in two years.

In order to fulfill that commitment, ASHI convened a Transplant Histocompatibility Conference Follow-up meeting in St. Louis on November 7, 2010 to gather and organize data needed to answer the concerns identified by the first Transplant Histocompatibility Conference. Participants who were drawn from the National Affairs Committee, the Scientific and Clinical Affairs Committee, UNOS, ASHI Executive Committee and invited presenters included: Debbie Crowe (Moderator), LeeAnn Baxter-Lowe, Patricia Campbell, Marcelo Fernandez-Vina, Lorie Gore, Amy Hahn, Malek Kamoun, Geof Land, Susie Leffell, Karen Nelson, Harriet Noreen, Allen Norin, Carol Pancoska, Donna Phelan, Elaine Reed, Nancy Reinsmoen, Peter Stastny, Anat Tambur and Dolly Tyan. Additional members will be added to the Task Force groups as the projects progress.

In the morning session, the participants heard presentations aimed at identifying as many issues as possible that can influence solid phase HLA antibody testing and complicate the interpretation and analysis of the test results and comparison of data.

Dr. LeeAnn Baxter-Lowe set the stage for the meeting by presenting a summary of the HLA antibody testing literature published since the THC in 2008. The articles that she cited posed a number of issues that need to be addressed by the group:

- How to address donors whose alleles may not be represented on the beads
- Standardized reporting using MFI, MESF, ratios, sum of MFIs for multiple DSAs
- Minimizing technical variation
- Publication criteria for description of reagents, lots, methods and definition of cutoffs
- Identifying what matters: levels/changes in levels; cumulative effects, locus-specific effects, donor alleles/epitopes, B cells/plasma cells
- What factors decide AMR/outcome - DSA with no AMR vs DSA with AMR; former have better outcome although there are no differences in donor antibodies.
- The role of the immunologic memory response in increased risk from weak pre-transplant DSA
- Standardized approach to post transplant antibody measurements: rising Ab levels lead to AMR, falling Ab levels no AMR

Dr. Susie Lefell presented data from Dr. Nancy Reinsmoen, Dr. Andrea Zachary and herself describing the effect of lot-to-lot variations and interfering substances in solid phase immunoassays (SPIs) for HLA specific antibodies. The sensitivity of SPIs makes them more susceptible to variation and interference, including:

- Denaturation and conformational changes in target molecules
- Variation in amount of target antigen on beads.
- Non-specific binding
- Lot- to- lot variation; recent deliberate increase of antigen on beads by one of the vendors to increase sensitivity
- Interfering serum substances such as high levels of IgM blocking antibodies, immune complexes, antibodies to plastics or latex, therapeutic antibodies. Clues are low positive controls, high negative controls or reactivity with autologous antigens.

She recommended several areas in which the group could move forward:

- Consistency in flow cytometric crossmatch reporting (MCS, MESFs, MCF, ratios, normalized values)
- Consistency in SPI procedures, validation, performance, interpretation and reporting
- Educational initiative

Dr. Leffell went on to say that an educational initiative by ASHI may yield immediate improvement in reducing variation in HLA antibody testing results by ensuring that all labs are using and interpreting SPIs appropriately. She identified several topics to be considered by all laboratories using SPIs for HLA antibody testing: the weaknesses and pitfalls in SPIs, the value of using more than one assay, the importance of controls and when they indicate interference, appropriate validation and ongoing QA, and the problems caused by reporting MFI values without clinical correlations, reference ranges or normalization.

Dr. Peter Stastny discussed the experience of UT Southwestern Medical School using Normalized Ratios to determine an accurate cutoff for single antigen beads.

Normalized Ratio (NR) = [(Raw MFI x Ag density correction factor) – NC)]/ NHS Mean + 3 SD

- NR of > 2x the cut-off is considered positive. Above 10x is a strong positive
- Cut off is 2 x (mean + 3SD) using "Normal Human Sera" after eliminating outliers
- Normalized ratios method has a different cutoff for each bead.
- Normalized ratios yielded more reproducible results when comparing sequential serum samples than normalized MFIs (raw MFI – the negative control for the bead).
- Normalized ratios generally identified more weak positive specificities than normalized MFIs.
- Specificities that were identified with normalized ratios but were negative when using a fixed MFI cutoff, could yield a positive crossmatch.
- Normalized ratios correlated with crossmatch and biopsy (C4d staining +) better than normalized MFIs.

Dr. Stastny enumerated several issues to address in the use of Normalized Ratios:

- Monoclonal antibodies for standardizing assays, especially those specific for DQ and DP, are hard to find
- Antigen Density on the beads is important
- Reactions of normal sera can be highly variable
- Methods used to analyze single antigen tests can impact the antibody specificities.

A computer program for the calculation of NR is available at:
http://www4.utsouthwestern.edu/stastny-lab/SAG%20ANALYSIS/Download_Page.htm

Dr. Deborah Crowe described her experience using a calculated Relative Ratio to establish a cut-off. Her data suggests using Relative Ratios as an alternative for MFIs which have run-to-run and tech-to-tech variations. The range of MFI values is also very large and makes it difficult to establish the significance of small changes in value. The manufacturer of one kit suggests using a normalized background ratio to establish the cutoff. A normalized background (NBG) ratio is calculated as follows and can be included on the results printout along with the MFI of each bead in the assay.

NBG ratio = _____Test bead/Test bead Neg_____
                    Neg serum control/Neg serum control Bead Neg

Normalization against the internal negative control helped to correct for background and normalization against the negative control serum helped correct for run-to-run variations. While the NBG ratio can be used to establish the cutoff, that number differed greatly between samples. To standardize this number, she compared the NBG ratio with the Pos/Neg ratio and calculated a Relative Ratio proportional to the Pos/Neg ratio being arbitrarily set at 100.

Relative Ratio = ____Test NBG ratio (100)____
                         Pos/Neg ratio

Dr. Crowe was able to show that, when using the same lot of reagents, the Relative Ratio was stable run-to-run and tech-to-tech. The results are consistent over time with repeat specimens while the MFIs are more variable. There were some limitations to using relative ratios: RR works best if P/N > 20, high background needs to be resolved before testing and a consistent calibrator is needed. She was also able to establish a Relative Ratio that correlated with a positive flow crossmatch. This relative ratio was set as the cutoff when identifying clinically significant antibodies. Comparison of PT results between two laboratories showed excellent correlation of Relative Ratios for the specificities identified.

Dr. Crowe identified several issues for the group to address:
- It may be possible to standardize the NBG ratio against a calibrator (in this study, the positive control was used as a 'calibrator'). If we could find a suitable calibrator that can be used by all labs and for all lots, it might be possible to establish a stable Relative Ratio across all labs to provide a common basis to compare results lab to lab.
- A cutoff value for the Relative Ratio can be established that correlates with a positive crossmatch.
- The Relative Ratio also could be used to monitor the level of antibody post-transplant

Dr. Karen Nelson spoke about the development of a consistent calibrator for use in HLA antibody testing. She described the ideal characteristics as follows:
- Control formulated from IgG monoclonals (human) which are consistent over time
- Standardized with grams of protein
- Vendor independent or cooperative initiative
- Inverse of NC1
- Important for multiple labs working on an HLA antibody testing project

She noted that Ilias Doxiadis has a prototype that should be considered by of one of the working groups

Dr. Elaine Reed discussed the NIH Initiative to standardize HLA antibody testing for the Clinical Trials in Organ Transplantation (CTOT) project. She described the CTOT experience as a model for standardizing methods to be used by multiple laboratories working cooperatively on a project. The CTOT aims are as follows:

Aim 1: Develop standardized antibody testing methods for HLA and MICA across CTOT core labs
- Unify testing platform and methods, cross-validate manual vs. automation, and cross-validate different vendor reagents (strictly using the vendor package inserts)
- Develop Standard Operating Procedures for assay performance, control values and test failure

Aim 2: Develop uniform, robust analysis strategies for HLA and MICA antibody testing and data archive capabilities
- Standardize antibody quantification (normalized values, MFIs) and establish uniform criteria for negative cutoff values
- Determine the correlation between luminex MFI and lymphocyte crossmatches
- Electronic transmission of results to centralized data archive for analysis

Aim 3: Establish validated test reagents for HLA and MICA antibody testing across CTOT clinical trials

- Secure validated reagents for HLA and MICA antibody testing for prospective CTOT studies
- Partner with commercial vendor to ensure lot-to-lot uniformity in luminex reagents, allele representation and bead antigen density.

Aim 4: Develop HLA and MICA reference reagents for continuous assay validation
- Develop shared resource for reference reagents for HLA antibody and MICA antibodies to QC/validate assays

Aim 5: Develop a Quality Assessment program for the CTOT core laboratories
- Use of reference sera to validate assay performance and tech competency, blind sample testing, and establishment of an inter-laboratory proficiency testing program

Aim 6: Implement a laboratory training program for assay standardization and data interpretation
- Train laboratory staff in assay performance and data interpretation, publish methods for scientific community, provide web-based access to SOPs for the scientific community

Aim 7: Develop a repository of clinical trial samples for future validation and research studies.
- Develop standard operating procedures for sample collection times, specimen type and specimen storage (aliquots), labeling, and freeze/thaw criteria

Dr. Malek Kamoun discussed the challenges in assessing anti-HLA DQ alloantibody strength and specificity. He described a study in which six sera with anti-DQ specificity, but no class I or DR specificity, were evaluated using a Luminex single antigen bead (SAB) assay and flow cytometric crossmatches using a surrogate cell panel selected for DQ type. The results showed a poor correlation between the MFI values of anti-DQ antibody reactivity observed in the SAB assay and the MESF values of antibody reactivity with B cells using a flow cytometry crossmatch. Dr. Kamoun stated that MFI values reflect antibody avidity and not only antibody concentrations. Discrepancies of antibody reactivity were observed in both directions (strong reactivity by SAB vs. very weak reactivity by FCXM and weak reactivity by SAB vs. strong reactivity by FCXM). In several cases, an anti-DQ antibody specific to a shared DQ epitope (such as DQ7,8,9) showed over 5-fold difference in antibody reactivity with B cells from various subjects carrying the same DQA1/DQB1 alleles.
He outlined some factors that may affect the results of DQ antibody identification:
- HLA-DQ determinants that may affect anti-DQ antibody avidity include:
  a. Polymorphisms at HLA-DQ-alpha amino acid sites
  b. Polymorphism at HLA-DQ-beta amino acid sites
  c. Structure of the peptide displayed by the DQ-alpha/DQ-beta heterodimer molecule
- Factors that may affect antibody reactivity in the Luminex SAB Assay may include:
  a. Cross-inhibition due to competitive binding among various DQ beads carrying shared epitopes
  b. Bead saturation- Dilution of sera can reveal a "prozone" effect
  c. Blocking factors/IgM antibodies revealed by serum treatment with DTT
  d. Some reactivity may be due to denatured epitopes (false positive)
  e. Interfering substances in patients' sera including: circulating immune complexes, platelet membranes/clotting, and sepsis
  f. The concentration of DQ antigens on SAB is substantially greater than that of other antigens, such as DR.

- Factors that may affect the surface expression of DQ-alpha/DQ-beta heterodimer molecules may include:
  a. Variation secondary to cis vs. trans molecular associations between different DQA1 and DQB1 allele products.
  b. Transcription regulation: allele/haplotype specific.
  c. Allele-dependent alternative splicing leading to soluble HLA-DQ-beta chains

Dr. Kamoun concluded that:
- Anti-DQ antibody reactivity level determined by SAB assays may not accurately reflect the level of reactivity with cell surface DQ-alpha/DQ-beta molecules.
- Possible allele/haplotype-specific variations in the level of surface expression of DQ-alpha/DQ-beta heterodimer molecules (variation range may be over 5-fold).
- Cell–specific structural variations secondary to the nature of the peptide captured by the HLA DQ-alpha/DQ-beta molecule may influence the reactivity of anti-DQ antibodies.

He posed several questions for the group to consider:
- What is the clinical significance of circulating anti-DQ DSA in the absence of graft dysfunction or pathological evidence of AMR?
- Could antibodies to certain epitopes be more linked to graft failure than others?
- Should the clearance of anti-DQ antibodies be used as a primary endpoint to assess the efficacy of desensitization treatments?

Dr. Dolly Tyan discussed the utility of HLA antibody testing methods that measure IgG vs C1q binding.
- Class I and Class II antibodies have a disparity in the ability to bind C1q
- C1q binding is highly associated with rejection and graft loss if the DSA is produced de novo
- At her center, transplants are not done if DSA is C1q positive pre-transplant

She concluded that IgG MFI is not as predictive of imminent graft rejection as C1q. As long as the C1q binding is negative, the patient is not in trouble. A question that she left for the group to consider: Should HLA antibody testing be performed exclusively using IgG or should C1q binding be assayed also?

In the final formal presentation, Dr. Marcelo Fernandez-Vina described epitope identification and the effects of shared epitopes on HLA antibody specificity identification. He summarized his presentation as follows:
- The computational approach identified 192 HLA-A and B epitopes; these were confirmed by 274 monoclonal antibodies (50 were identified by more than one MAb). 73 epitopes on HLA-A alelles, 117 epitopes on HLA-B alleles, 2 epitopes on both HLA-A and B
- Most epitopes include multiple residues.
- 21% of the mapped epitopes include at least one residue buried in the peptide-binding groove.
- All 'groove epitopes' involve at least one additional residue in the alpha-helix connecting loop.
  o Some MAb may recognize tri-molecular complexes with a particular spatial configuration.
  o The lack of serologic distinction between alleles with differences in the peptide binding groove may have resulted from variability in detection due to operationally poor typing reagents.
- Some epitopes involved three or four polymorphic areas of the molecule

- Some patients make antibodies recognizing these complex epitopes (e.g. Bw4 like epitopes that do not react with HLA-B13) in spite of carrying all eplets or triplets on different molecules.
- In addition to some of the private epitopes of HLA C, a diallelic system that corresponds to KIR ligand groups of HLA-C has been described. These epitopes correlate with the dimorphism at the residue 80 of HLA-C
  - One of the epitopes includes extra inter-locus reactivity with HLA-B46 and B73
  - This supertypic epitope coincides with the sequence present in HLA-B alleles positive for Bw6 except that substitutions present at residue 76 distinguish HLA-C alleles from HLA-B except HLA-B46.
  - HLA-B46 is a ligand for KIR receptors 2DL2 and 2DL3 that also bind HLA-C alleles.
- Class II antibodies identified 60 DRB1 epitopes and 19 DQ epitopes.
- Application in solid organ transplantation
  - Perform HLA typing with SSO reagents that can differentiate common and well documented (CWD) alleles and sort genotypes into 3 categories: CWD-CWD, CWD-rare, rare-rare.
  - List likely genotype
  - Assign serologic equivalent for the likely genotype using 'virtual serology"
  - Perform virtual crossmatch based on epitopes

Dr. Fernandez-Vina identified a number of areas open for discussion/investigation:
  - Naming epitopes present in single and multiple loci, identifying corresponding alleles; naming epitopes defined by one and two subunits in class II; naming new serologic specificities for loci without current serologic equivalents.
  - Novel sensitive methodologies allow for precise epitope mapping
  - Extensive HLA typing of the patient is important to exclude self epitopes
  - Analysis of epitopes present in the donor, quantitation of MFI and levels of antigen expression may help in the prediction of cross-match results.
  - In some instances the solid phase assays cannot predict the cross-match results, because in the former there may be competition for antibodies recognizing epitope(s) present in multiple antigen preparations
  - 'Virtual serology'- predicting the reactivity of new alleles; epitope analysis may help predict cross-match results for antigens not present in the screening panels
  - HLA matching algorithms
  - Epitope immunogenicity studies
  - Next challenge: non-HLA antigens, tissue-specific antigens

The issues identified in the presentations could be grouped into several broad categories: variability in reagents, techniques, analysis and descriptions of methods for data used in publications; biological hurdles; epitope definition and analysis; and clinical correlation.

Using the issues identified earlier in the day, the participants spent the second half of the meeting forming work groups to address approaches that could be employed to address these issues.

WORK GROUPS, STRUCTURE AND SCOPE

I.   Variability Issues working group
      A.   Reagents
      B.   Techniques
      C.   Analysis
      D.   Publications- criteria for method descriptions

      Team Leader: Deborah Crowe
      Members: Elaine Reed
                     Karen Nelson
                     LeeAnn Baxter-Lowe
                     Peter Statsny
                     Anat Tambur
                     Geof Land
                     Harriet Noreen
                     Donna Phelan

II.   Biological Hurdles working group
      A.   HLA shared epitopes: Effects of antibody affinity and competitive binding on MFI values
      B.   HLA-DQ expression on B cells
      C.   Anti-HLA antibody Ig subclasses
      D.   Effects of various treatments on Luminex assays
      F.   Interference from IgM, immune complexes, etc.

      Team Leader: Malek Kamoun
      Members: Amy Hahn
                     Geof Land
                     Carol Pancoska
                     Jim Cicciarelli

III. Clinical Correlation working group
      A.   Effect of complement fixing antibodies
      B.   Correlation of antibody concentration and crossmatch results
      C.   Correlation of antibody concentration and transplant outcome
      D.   Correlation of antibody parameters (concentration, complement fixing, subclass,) with
            pathology
      E.   Molecular biomarkers
      F.   Differences in correlation with different organ groups (kidney, heart, lung, etc)

      Team Leader: (TBD)
      Members: Lee Ann Baxter-Lowe
                     Susie Leffell
                     Dolly Tyan
                     Patricia Campbell
                     Peter Statsny
                     Carol Pancoska

IV.   Epitope Definitions and Analysis working group
        A.   Eplets vs. Terasaki definition vs. Marcelo's definition
        B.   IDAWG
        C.   Missing alleles in panels

        Team Leader: Marcelo Fernandez-Vina
        Members: Lee Ann Baxter-Lowe
                        Susie Leffell
                        Allen Norin
                        Malek Kamoun
                        Dolly Tyan
                        Anat Tambur

ORGANIZATION, STRUCTURE AND RESPONSIBILITIES OF THE WORK GROUPS

1. The steering committee will initially be those who attended the 11/7/10 meeting.
2. Each group had a breakout session to choose a Team Leader, design projects, prioritize projects, make assignments, propose other ASHI members to bring into task force to work on projects.
3. Each group was to establish milestones or objectives and set timelines for each project to be completed.
4. The Team Leader is responsible for ensuring that progress is being made to meet deadlines.
5. The Team leader is responsible for preparing a quarterly report.
6. Quarterly report will be sent to all members of the Steering committee to identify overlap between the groups. The findings of one group may be important for other groups.
7. Communication between groups will occur by using an online bulletin board that will be set up by ASHI. All progress notes and communication should be posted on the bulletin board. All members of the task forces should have access to each other's notes and communications.
8. The bulletin board will not be accessible to all ASHI members at this time.
9. After the quarterly reports are reviewed by all members of the Steering Committee, a progress report will be made combining the reports from each group. This report will be made available for all ASHI members through ASHI web site and/or ASHI Quarterly.

Those interested in working with a specific Task Force should contact the Team Leader. We encourage participation by as many laboratories and transplant centers as possible to strengthen this collaborative effort.