Transplant Applications of Solid-phase Immunoassays

Anti-HLA antibody testing in solid organ transplantation

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28 April 2015

Objectives

1. Appreciate the critical role of histocompatibility laboratory testing in solid organ transplantation.

2. Understand antibody testing methods used to assess immunologic risk prior to transplantation and to diagnose rejection after transplantation.

3. Understand challenges in antibody test interpretation and laboratory medicine consultation.

NO DISCLOSURES
Outline

1. Introduction to Transplantation
2. The HLA laboratory in Transplantation (case presentation)
3. Methods for anti-HLA antibody testing
4. Challenges in test interpretation and clinical consultation
5. Pop Quiz!

Introduction to Transplantation

• Different types for end-stage organ dysfunction
  kidney, liver, heart, lung, pancreas, intestine, VCA

• Donors: deceased or living but...

DEMAND >>> supply

<table>
<thead>
<tr>
<th></th>
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<th>Transplanted (in 2014)</th>
<th>Median wait (blood group O)</th>
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<tbody>
<tr>
<td>All</td>
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Introduction to Transplantation

• Why transplant?
  - advantages of transplantation (over dialysis)
    a) improved quality of life
    b) improved survival
    c) cost savings

| Table 2. Annual Death Rates and Total Numbers of Death, 1991 – 1997.1 |
|-----------------|-----------------|-----------------|-----------------|
| Variable        | All patients   | Patients on the Waiting List | Patients on the Waiting List + Deaths |
| Age             | Total in 0/1000 | Total in 0/1000 | Total in 0/1000 |
| 0-19 yr         |                  |                  |                  |
| 20-39 yr        |                  |                  |                  |
| 40-59 yr        |                  |                  |                  |
| >60 yr          |                  |                  |                  |

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Introduction to Transplantation

- 10-year savings for renal transplantation (vs life-long dialysis)\(^1\)
  
  **Dialysis:** $500,000  
  **Transplant:** $150,000  
  
  $50,000 annually  
  $65,000 one-time cost  
  $8,500 annually  
  
  **Net savings:** $350,000 per patient

<table>
<thead>
<tr>
<th>Cost Description</th>
<th>Dialysis Cost Savings</th>
<th>Government Cost Savings</th>
<th>Social Cost Savings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis Loss of Transplant Maintenance  (^1)</td>
<td>42,588</td>
<td>42,588</td>
<td>42,588</td>
</tr>
<tr>
<td>Disability payments  (^2)</td>
<td>15,000</td>
<td>15,000</td>
<td></td>
</tr>
<tr>
<td>Lost taxes from not working  (^3)</td>
<td>5,000</td>
<td>5,000</td>
<td></td>
</tr>
<tr>
<td>Patient out-of-pocket costs  (^4)</td>
<td></td>
<td>6,000</td>
<td></td>
</tr>
<tr>
<td>Lost wages  (^5)</td>
<td></td>
<td>36,499</td>
<td></td>
</tr>
</tbody>
</table>

**Annual Cost Savings per Patient:** $423,084  
$60,909  
$83,087

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Basic Transplant Immunology

**Assumptions:**

- Immune system functions to protect us from non-self (and abnormal self, i.e. cancer).
- This function is a major impediment to transplantation

- The Human Leukocyte Antigen (HLA) system is central to determining ‘self’ versus ‘non-self.’

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Basic Transplant Immunology

How then can transplanted, donor tissue exist in a recipient?

1) Recipient = allograft donor (monozygotic twins)
2) Recipient lacks the immune armament (against the graft)
   - there is central or peripheral tolerance
   - HLA-matched (fully)
3) Recipient is given immunosuppression
   - most transplant patients fall in this category
   - major issue of drug-related toxicities (infections, organ damage, cancer risk)
The HLA Laboratory

- Role is to test, interpret and provide information that:
  1) Minimizes risk of rejection (acute and chronic)
  2) Assists in determining if clinical event is rejection

- 2 broad categories of tests
  1) Typing - patient (recipient) and/or donor
     a) HLA
     b) non-HLA determinants - minimal
     c) Disease association (e.g. B27)
     d) Chimerism testing – for stem-cell transplant
  2) Assess alloreactive response
     a) Donor specific antibody
     b) T-cell response

The Human Leukocyte Antigen (HLA) System

- multiple genes
- chromosome 6
- inherited as haplotype (one from each parent)
- structural genes: Class I and II

Class I
  - all nucleated cells
  - 3 sets: HLA-A, -B and -C

Class II
  - present on select cells
  - 3 main: HLA-DR, -DQ and -DP

HLA Class I and Class II

- Class I molecule
  - encoded by single gene
  - all individuals inherit one allele from each parent

- Class II molecules – DR, DP, DQ
  - encoded by 2 genes
  - individuals in theory, could have:
    6 different molecules/antigens*
    12 different genes*
*actually – it is 8 molecules & 14 genes bef of DR3/4/5)
How immunogenic are allogeneic HLA antigens?

- HLA molecules are very immunogenic
- Allogeneic exposure:
  1) Pregnancy
     - ~24% of previously pregnant women have HLA Ab
     - allosensitization increases with parity
  2) Transplantation
  3) Transfusion
     - 12-17% HLA sensitization due to transfusion
     - 2% individuals with remote transfusion (up to 10 years)

<table>
<thead>
<tr>
<th>Number of Pregnancies</th>
<th>% with anti-HLA Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11%</td>
</tr>
<tr>
<td>2</td>
<td>22%</td>
</tr>
<tr>
<td>3</td>
<td>27.5%</td>
</tr>
<tr>
<td>4</td>
<td>32%</td>
</tr>
</tbody>
</table>

Antibodies in Allograft Rejection

- Allograft rejection – initially thought to be T cell-mediated process
- Landmark study in 1969
  - Patel & Terasaki
  - Hyperacute rejection in patients with pre-formed HLA Abs
  - reason for testing pre-tx
- Recent incidence of HLA Abs
  - pre-Tx: ~20% has PRA>10%
  - post-Tx: 30% de novo DSA

Antibody-Mediated Rejection (AMR)

- The role of Donor Specific Antibodies (DSA) is well accepted
- What is Donor Specific Antibody
  - antibody recognizing allogeneic HLA antigen
  - but can also be against non-HLA target
  - 2 pieces of information needed for DSA:
    a) specificity of antibody
    b) donor HLA type
- A clinicopathologic diagnosis
  1) Graft dysfunction
  2) Tissue injury
  3) Evidence of antibody activity (Ig deposits or complement)
  4) Circulating DSA
Renal Histology of Acute AMR

Histologic features: capillaries with PMN/macrophages, platelet thrombi and necrosis, C4d+

Regulatory Aspects of Transplantation

- Health and Human Services (HHS) and Centers for Medicare and Medicaid Services (CMS)
- Organ Procurement and Transplantation Network (OPTN)
  - maintains the nationwide registry/database
  - develops policies for transplantation
- 42 CFR 193.1278 Standard: Histocompatibility
  1) Antibody screening – method specificity > basic CDC
  2) Crossmatch (XM) – method sensitivity > basic CDC
  3) Kidney transplants - final XM result prior to transplant

  Makes histocompatibility testing complex

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The Role of the Histocompatibility Laboratory in Transplantation

Case 1: Pre-operative assessment

• A 21 y.o. woman with HIV nephropathy
  • 'Healthy' until pregnancy at 16 y; did not receive pre-natal care; diagnosed with HIV infection during L&D
  • Patient put on HIV therapy (HBV- and HCV-negative)
  • Developed HIV nephropathy over the last 5 years (unclear compliance but no history of AIDS-defining infections and CD4 count was 696/µL)
  • Now being seen for kidney transplant (living donor, cousin)

The Role of the Histocompatibility Laboratory in Transplantation

Case 1: Pre-operative assessment

• Initial testing
  a) Donor typing: A1, A68, B35, B61
     DR4, DR7, DO2, DO8

  b) Patient serum positive for antibody against:
     A31, B27, Cw1, Cw17

  c) Crossmatch (patient serum x donor lymphocytes)
     (i) cytotoxicity (CDC): POSITIVE
     negative after DTE-treatment of serum
     (ii) flow cytometry (FCXM): POSITIVE

The Role of the Histocompatibility Laboratory in Transplantation

Case 1: Pre-operative assessment

• Incongruent histocompatibility testing results
  - cellular assays = NOT compatible
  - solid-phase assays = compatible

• Is this donor-recipient pair compatible?
  - you want to minimize risk of rejection because recipient is young
  - allograft survival: ~10 (deceased) to 15 (living) years
The Role of the Histocompatibility Laboratory in Transplantation

Case 2: Post-operative assessment

- A 60 year-old man with ESRD due to DM and HTN; on dialysis
- Was called in for deceased donor kidney transplant
- Patient is HLA allosensitized (i.e. he has many anti-HLA antibodies) but no apparent DSA to donor by solid phase assays; crossmatches are negative = COMPATIBLE
- Allograft offer accepted; transplant surgery uneventful

The Role of the Histocompatibility Laboratory in Transplantation

Case 2: Post-operative assessment

- POD#3-5: there is delayed graft function (not making enough urine and Cr is not decreasing)
- Post-tx testing:
  - by solid phase, still no DSA
  - but Flow crossmatch now VERY POSITIVE
- Is this an antibody-mediated process?
- Management of graft dysfunction depends on cause

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How do we test for anti-HLA antibodies?

• Methods
  1) Cell based – cells expressing HLA antigens (crossmatch, XM)
     a. Complement-dependent cytotoxicity (CDC) – ca. 1960s
     b. Flow-cytometry
  2) Solid-phase immunoassay (SPI) – purified HLA antigens
     a. ELISA - ca. 1990s
     b. Flow cytometry – ca. late 1990s-early 2000s
     c. Luminex - ca. mid-2000s

• Issues in testing
  1) Sensitivity
  2) Specificity
  3) Clinical significance
**Flow Cytometry**

- Anti-HLA Ab (recipient) plasma
- Test cell usually from donor
- Anti-IgG/HTC
- anti-CD3/PE

**Enzyme-linked immunosorbent assay (ELISA)**

- Can come as panel (mix of HLA molecules) or single antigens
- Some manufacturers detect both IgG and IgM

**Luminex Testing**

- Anti-HLA Ab (recipient) plasma
- Fluorescent bead
- HLA molecule
- anti-IgG/PE

**Readout:** MFI

- MFI
- Fluorescence intensity by HLA specificity

- Perhaps the most sensitive method for detecting HLA-antibodies
- Concern of being too sensitive and lacking "pathologic relevance"
3 Types of Luminex Testing

<table>
<thead>
<tr>
<th>Type</th>
<th>Molecules</th>
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<tbody>
<tr>
<td>Mix</td>
<td>HLA molecules &gt; 2 cell lines</td>
</tr>
<tr>
<td>Phenotype (PRA)</td>
<td>All class I or II from ONE individual</td>
</tr>
<tr>
<td>Single Antigen</td>
<td>Single protein (recombinant)</td>
</tr>
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Luminex – phenotype/PRA

Each bar = 1 bead color

Mean Fluorescence Intensity (MFI) = relative strength of antibody

Luminex – single antigen

Each bar = 1 bead color

Mean Fluorescence Intensity (MFI) = relative strength of antibody
Pros and Cons

- **Cell-based** non-HLA targets detected, need viable cells
  1) CDC  best indicator of incompatibility  
     most technical of methods; most variable  
  2) Flow  2nd best for risk of incompatibility  
     also variable, but more quantitative than CDC  

- **Solid-phase (SPI)** very sensitive; can define specificity, cost  
  1) ELISA  rapid, can be semi-quantitative  
  2) Luminex  best to ID antibody specificities in  
     highly-sensitized pt; virtual crossmatch;  
     better for identifying DQA1 and DP antibodies  
  3) Flow  similar to Luminex but less multiplex  

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So which test should we use?

- Ideally – 100% sensitive and 100% specific  

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<th>Recipient</th>
<th>CDC</th>
<th>Solid phase</th>
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<tr>
<td>Previous pregnancies alone</td>
<td>4</td>
<td>24-33</td>
</tr>
<tr>
<td>Prior transfusions alone</td>
<td>40</td>
<td>52</td>
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- Sensitivity:  
  CDC < AHG-CDC = ELISA < Flow < Luminex  

- Cytotoxicity – still best for clinically significant DSA  
  (i.e. if true positive, no one will transplant)  

- Recommended that you do both cell- and solid phase-based  
  testing²

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²Am J Transplantation (2011) 11:1785-1791  
²Transplantation (2013) 95:19-47
Known Interferences in HLA Antibody Testing

- Endogenous to patient
  a) High-titer anti-HLA antibodies – prozone effect
  b) Complement &/or IgM – blocking (false negative)
  c) Autoantibodies – false positive (CDC, FCXM and SPI)
  d) Non-specific antibodies – neoantigens on solid-phase
- Iatrogenic
  a) Rituximab (in recipient serum) - can bind donor lymphocytes and cause false positive B cell FCXM
  b) ATG can cause false positive cellular assays (the ATG in recipient serum attacks donor lymphocytes); ATG can cause false negative solid-phase
  c) IVig can cause a number of problems (high background) or block (false-negative CDC via complement or anti-idiotype)

Challenges with SPI Antibody Testing

- Subject to interferences plaguing immunoassays!
  1) Identifying HLA antibodies - is it real?
  2) Assigning clinical relevance to HLA antibodies
     - strength (MFI, reactivity score), titer
     - when should we be testing? how often?
     - complement fixing: C1q, C4d, other
     - isotype and subclass (IgG1/3 vs IgG2/4)

- Cost - #70 in 2014 CMS reimbursement!

Is it real? Yes, no, maybe, perhaps

- Sensitized patient - 2nd transplant
- Potential donor is HLA-A1 positive
- Patient now has a ‘new’ A1 antibody
- Need ‘perfect’ donor – recipient has poor absorption (cannot tolerate too much immunosuppression)
- Cellular crossmatch with surrogate donor A1 cells: NEGATIVE
- Test with phenotype bean SPI: NEGATIVE
- A1 antibody is probably ‘not real’
**Masking?**

- Treatment with DTE removes IgM

**Sometimes, you just have to give up...**

- NEGATIVE CTRL BEAD – VERY HIGH!!
- Patient got ATG and IVIg...

**Alloantibodies Are NOT Made Equal**

- Patel and Terasaki - 1969
  - role of anti-HLA antibodies in hyperacute rejection

- However, rejection can still occur even without anti-HLA antibodies¹
- Rejection also does not always occur even with cytotoxic-positive²

- What about allograft factors: accommodation?
  - low-dose anti-HLA antibodies might ‘protect’ allograft by:³
    a) ↑Bcl-XI on endothelium
    b) no effect on adhesion molecule expression
    c) susceptibility to complement-mediated death

¹NEJM (1970) 278:642-648
³Am J Transplant (2001) 1:260-269
Currently, thrust of research and development efforts to identify these 2 stages of antibody-mediated injury earlier

Non-HLA Antibodies in Transplantation

- 10% of C4d+ kidney rejection not due to anti-HLA antibodies
- Decreased survival of kidney allografts in HLA-identical sibling pairs - role of antibodies against non-HLA targets
- Some non-HLA allo-targets implicated:
  - Endothelial cell antigens - MICA/B, HY - slight increased risk of graft failure
  - ABO and other non-ABO red cell antibodies
- Some auto-targets:
  - ANCA
  - AT1R
  - Epithelial cell antigens
  - Structural: tubulin, vimentin, laminin

The Role of the Histocompatibility Laboratory in Transplantation

Case 1: Pre-operative assessment

- Is this donor-recipient pair compatible?
  - Yes, they are compatible: no DSA by Luminex, cellular assay reactivity attributable to antibodies that are not of significance
  - Post-transplant doing well

Case 2: Post-operative assessment

- Is delayed function due to antibody?
  - Management of delayed graft function depends on cause
  - No, this is not antibody-mediated rejection: Luminex — no DSA, but Flow XM positive probably due to ATG!
Take Home Points

• Histocompatibility testing crucial for pre- and post-transplant management

• HLA antibodies are key in getting AND keeping your allograft

• Laboratory expertise needed to interpret testing results
  - remember not every antibody is real
  - not every negative result is negative

• Clinical expertise needed for consultation (clinical significance of antibody)

Pop Quiz!

1. Which of the following is a risk factor for HLA alloimmunization?
   a) blood transfusion
   b) pregnancy
   c) transplantation
   d) trauma

2. Which is NOT needed to establish compatibility between potential recipient and donor pair?
   a) Recipient HLA typing
   b) Donor HLA typing
   c) Recipient anti-HLA antibody testing
   d) Donor anti-HLA antibody testing

Pop Quiz!

3. The 2 main types of antibody assays are cellular and SPI. It is recommended that both an SPI and cellular assay be performed. Which 2 methods meet this recommendation?
   a) AHG-CDC and Flow cytometry
   b) Luminex and ELISA
   c) AHG-CDC and Luminex
   d) Flow cytometry and ELISA

4. Which of the following can cause false-negative interference in SPI which can impact interpretation of results and result in transplantation of an incompatible organ?
   a) Rituximab
   b) IgM
   c) ATG
   d) Complement
Pop Quiz!

5. Post-renal transplant, a recipient’s serum Cr begins to rise. By SPI, the patient now has a new anti-HLA-A2, A23 and A24 antibodies. To make the diagnosis of antibody-mediated rejection, donor specific antibody (DSA) must be present. What must be true for DSA to be present?
   a) Donor HLA type is: A1, A3, B7, B8, DR4, DR9
   b) Donor HLA type is: A1, A24, B13, B44, DR1, DR17
   c) Donor ABO type is: O positive
   d) No additional information needed

Acknowledgements

Beth Israel Deaconess Medical Center
Pathology
Transplant Institute

NIH/NHLBI – K12HL087164-07