Optimizing Solid Phase Assays

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Evolution of HLA antibody testing

- Cell based assays (T and B cell)
- Solid phase crossmatching
- Cytotoxic FCXM
- Multiplex suspension/chip arrays
- Flow cytometry (microparticles)
- Flow cytometric crossmatching (FCXM)
- Enhanced cytotoxicity (e.g., AHG)
- Cytotoxicity (NIH)
- Solid phase assays (class I/II)
- Screening identification

Sensitivity

Complement dependent

Complement independent

Patel and Terasaki. NEJM. 1969
HLA antibody identification by LABScreen® Single Antigen Bead Luminex Assay

100 different types of beads
Each bead type coded with different red/infrared dye combination
Each bead type is coated with different recombinant HLA antigen

Detection with anti-IgG-PE

Reporter laser (532nm)
Tells the instrument how much PE dye is bound to the bead

Classification laser (635nm)
Tells the instrument which bead is being examined
HLA antibody analysis
Patient with a history of transplant

Class I HLA

Unacceptable antigens:
A: 1 2 3 11 26 29 30 31 33 34 43 66 68 69 74 80
B: 7 13 27 47 48 60 61 73 81

Class II HLA

Unacceptable antigens:
DR: 1 103 7 9 15 16 51 53

Patient’s HLA typing A23,24 B44,56 DR17,13, DR52, DQ2,7
Significant decrease in graft survival in patients with pre-transplant DSA

Significant decrease in graft survival in patients with pre-transplant DSA

Lefaucher et al, *JASN 2010.*
Calculated Panel Reactive Antibody (cPRA)

Canadian cPRA Calculator

The Canadian cPRA calculator is a component of the Canadian Transplant Registry (CTR), a web-based application used by the transplant community, to estimate the percentage of Canadian deceased organ donors with whom a transplant candidate may be incompatible.

This calculator uses the same formula and data as the CTR. It produces a value by comparing the unacceptable antigens entered below. If you are a transplant candidate, and have questions regarding the value generated by this calculator, please contact your transplant physician or coordinator.

Attention: The actual cPRA value assigned to a transplant candidate is calculated by the CTR based on the unacceptable antigens that are entered by the laboratory at the transplant candidate’s transplant centre. The value produced by the cPRA calculator on this website is for your informational use only.

Highly sensitized

### Perception Versus Reality?: Virtual Crossmatch—How to Overcome Some of the Technical and Logistic Limitations

<table>
<thead>
<tr>
<th>Actual/FCXM</th>
<th>Positive</th>
<th>Negative</th>
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</thead>
<tbody>
<tr>
<td>Positive</td>
<td>515</td>
<td>83</td>
</tr>
<tr>
<td>Negative</td>
<td>83</td>
<td>854</td>
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</tbody>
</table>

- FP 3.1%
- FN 14%

- Some allele specific (non-DSA)
- Some weak DSA

- Non-HLA abs
- False pos FCXM
- Prozone

Sensitivity = 93.1%
Specificity = 85.3%

Tambur et al. AJT 9:1886, 2009
Calculated PRA: Initial Results Show Benefits for Sensitized Patients and a Reduction in Positive Crossmatches

J. M. Cecka\textsuperscript{a,*}, A. Y. Kucheryavaya\textsuperscript{b}, N. L. Reinsmoen\textsuperscript{c} and M. S. Leffell\textsuperscript{d}

Optn/Unos

Table 1: Number of positive crossmatches reported as a reason for organ refusal

<table>
<thead>
<tr>
<th></th>
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<tr>
<td>Not reported</td>
<td>589</td>
<td>677</td>
<td>588</td>
<td>0</td>
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<tr>
<td>0</td>
<td>3270</td>
<td>3155</td>
<td>5006</td>
<td>1288</td>
</tr>
<tr>
<td>1–20</td>
<td>2051</td>
<td>1800</td>
<td>1715</td>
<td>134</td>
</tr>
<tr>
<td>21–79</td>
<td>5735</td>
<td>5040</td>
<td>2477</td>
<td>565</td>
</tr>
<tr>
<td>80+</td>
<td>16,499</td>
<td>17,045</td>
<td>5,793</td>
<td>737</td>
</tr>
<tr>
<td>All</td>
<td>28,144</td>
<td>27,717</td>
<td>15,579</td>
<td>27,24</td>
</tr>
<tr>
<td>Deceased donor kidney transplants</td>
<td>4221</td>
<td>4197</td>
<td>5,295</td>
<td>4,953</td>
</tr>
</tbody>
</table>

10 fold decrease in positive XM
Improved allocation

Cecka et al. AJT 11:719, 2011
Calculated PRA: Initial Results Show Benefits for Sensitized Patients and a Reduction in Positive Crossmatches

J. M. Cecka\textsuperscript{a,\ast}, A. Y. Kucheryavaya\textsuperscript{b}, N. L. Reinsmoen\textsuperscript{c} and M. S. Leffell\textsuperscript{d}

Table 2: Deceased donor kidney transplants by era and recipient’s sensitization level

<table>
<thead>
<tr>
<th>Allocation PRA/CPRA group (%)</th>
<th>Era</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
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<tr>
<td>0/Not reported*</td>
<td>2732</td>
<td>64.7</td>
<td>2724</td>
<td>64.9</td>
<td>2905</td>
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<tr>
<td>1–20</td>
<td>837</td>
<td>19.8</td>
<td>820</td>
<td>19.5</td>
<td>902</td>
</tr>
<tr>
<td>21–79</td>
<td>349</td>
<td>8.3</td>
<td>346</td>
<td>8.2</td>
<td>743</td>
</tr>
<tr>
<td>80+</td>
<td>303</td>
<td>7.2</td>
<td>307</td>
<td>7.3</td>
<td>745</td>
</tr>
<tr>
<td>All</td>
<td>4221</td>
<td>100</td>
<td>4197</td>
<td>100</td>
<td>5295</td>
</tr>
</tbody>
</table>

*Patients with zero CPRA cannot be distinguished from those with no report, so these groups were combined for each time interval.

2 fold increase in HSP transplant
Center-Defined Unacceptable HLA Antigens Facilitate Transplants for Sensitized Patients in a Multi-Center Kidney Exchange Program

L. A. Baxter-Lowe¹, M. Cecka², M. Kamoun³, J. Sinacore⁴ and M. L. Melcher⁵

National Kidney Registry
91% concordance between virtual and FCXM
OPO Strategies to Prevent Unintended Use of Kidneys Exported for High PRA (>98% cPRA) Recipients

A. S. Paramesh, N. Neidlinger, M. Salvatore, A. Smith, A. Friedman, W. Payne, T. Taber and C. Wright

17/114 (15%) unexpected positive FCXM

Variability in listing unacceptable antigens
Allele specific abs
DQA and DP abs
Challenges with HLA antibody testing and virtual crossmatching

• Virtual crossmatch is only as good, as current, as accurate, and as complete as the HLA antibody and HLA typing information

• Reproducibility

• Interfering substances, “prozone” effect

• Antibodies against denatured HLA epitopes

• Interpretation
Reproducibility
Comprehensive Assessment and Standardization of Solid Phase Multiplex-Bead Arrays for the Detection of Antibodies to HLA

E. F. Reed¹,², P. Rao¹, Z. Zhang¹, H. Gebel², R. A. Bray², I. Guleria³, J. Lunz⁴, T. Mohanakumar⁵, P. Nickerson⁶, A. R. Tambrur⁷, A. Zeevi⁴, P. S. Heeger⁸ and D. Gjertson¹

facturers (AUC > 0.9) and suggested optimal cutoffs from 1000 to 1500 MFI. Global normalization further reduced MFI variation to levels near 20%. Standardization and normalization of solid phase HLA antibody tests will enable comparison of data across laboratories for clinical trials and diagnostic testing.

Figure 1: Laboratory variance for single antigen assays using standardized operating procedures versus nonstandardized procedures.
LABScreen® single antigen bead (SAB) Luminex protocol

• Incubate beads (5 µl) and serum 20 µl (RT) 30 min.

• Wash x3 (5 min/spin) 15 min.

• Incubate with 100 µl anti-IgG-PE, 1:100 dilution (RT) 30 min.

• Wash x2 (5min/spin) 10 min.

• Total assay time 1h 25 min.

Evidence for incubation time/reagent concentration? wash times?
Objectives

• To develop a rapid single antigen bead LABScreen protocol without compromising the sensitivity of the assay.

• Investigate the effects of:
  – Centrifugation time
  – Serum incubation time
  – Anti-IgG-PE incubation time
  – Serum volume
  – Anti-IgG-PE concentration

Liwski et al Hum. Immunol. 2017
Effect of reduced spin time

• Standard
  5 washes x 5 min  = 25 min
  1300 x g

• Rapid
  5 washes x 1 min  = 5 min
  1800 x g

Liwski et al Hum. Immunol. 2017
Effect of reduced spin time (1 vs 5 min) on bead counts

Class I beads

Class II beads

Bead count vs Bead number for Class I and Class II beads with 5 min and 1 min spin times, showing a comparison of bead counts between the two spin times. The legend indicates N=3.
Effect of reduced spin time

- **Standard**
  
  5 washes x 5 min  = 25 min
  
  1300 x g

- **Rapid**
  
  5 washes x 1 min  = 5 min
  
  1800 x g

No impact on bead counts or overall results
20 minutes saved!

Liwski et al Hum. Immunol. 2017
Effects of reduced incubation times

• Serum incubation time

• Anti-IgG-PE incubation time
Effects of reduced incubation time
¼ PPC, HLA class I

MFI

Bead number

30/30 min

Liwski et al Hum. Immunol. 2017
Effects of reduced incubation time
¼ PPC, HLA class I

Effects of reduced incubation time
¼ PPC, HLA class I

- Blue line: 30/30 min
- Red line: 15/30 min
- Green line: 30/5 min

Liwski et al Hum. Immunol. 2017
Effects of reduced incubation time
¼ PPC, HLA class I

Effects of reduced incubation time
¼ PPC, HLA class II

MFI

Bead number

Effects of reduced incubation time
Negative control serum

![Graph showing MFI (Mean Fluorescence Intensity) vs Bead number for Class I and Class II with different incubation times.](image)

Effects of reduced incubation time
NC and PC beads

NC bead (#1)

Small Effect on background

PC bead (#2)

Significant Effect on IgG binding

Serum/IgG-PE incubation time

Liwski et al Hum. Immunol. 2017
Conclusion

• Reduction in incubation time with serum and/or anti-IgG-PE results in decreased MFI values.

• Negligible impact on LSNC and NC bead reactivity.

• The degree of MFI decrease when incubation time with anti-IgG-PE was reduced was surprising.

• IgG-PE concentration appears to be sub-optimal?
Effects of increasing IgG-PE concentration ¼ PPC, HLA class I

![Graph showing the effects of increasing IgG-PE concentration on MFI. The x-axis represents bead number, and the y-axis represents MFI. Different lines represent different concentrations: PE 1/5, PE 1/10, PE 1/20, PE 1/50, and PE 1/100.](image)
Effects of increasing IgG-PE concentration ¼ PPC, HLA class II

Liwski et al Hum. Immunol. 2017
Effects of increasing IgG-PE concentration

Negative control serum

Class I

MFI

Class II

Bead number
Effects of increasing IgG-PE concentration
NC and PC beads

NC bead (#1)
- Negligible effect on background

PC bead (#2)
- Significant effect on positive rxns

Serum/IgG-PE incubation time

Liwski et al Hum. Immunol. 2017
Conclusion

• Increasing the anti-IgG-PE concentration from 1:100 to 1:5 increases MFI in the standard assay including PC bead MFI.

• Negligible effect on background (LSNC and NC bead).

• Improved signal to noise delta

• Can we compensate for reduced MFI values in the 15/5 min protocol by optimizing the concentration of anti-IgG-PE?

Liwski *et al* Hum. Immunol. 2017
Effects of increasing IgG-PE concentration on MFI in 15/5 protocol\textsuperscript{1/4} PPC, HLA class I

![Graph showing the effects of IgG-PE concentration on MFI.](image)

Effects of increasing IgG-PE concentration on MFI in 15/5 protocol\textsuperscript{1/4} PPC, HLA class I

Effects of increasing IgG-PE concentration on MFI in 15/5 protocol¹/₄ PPC, HLA class I

Liwski et al Hum. Immunol. 2017
Effects of increasing IgG-PE concentration on MFI in 15/5 protocol PPC, HLA class I

Effects of increasing IgG-PE concentration on MFI in 15/5 protocol for PPC, HLA class I
Effects of increasing IgG-PE concentration on MFI in 15/5 protocol¼ PPC, HLA class II

Liwski et al Hum. Immunol. 2017
Conclusion

- Increasing concentration of anti-IgG-PE compensates for the reduction in incubation times.

- IgG-PE concentration of 1:10 closely matches MFI obtained with the standard assay.
ROB LABScreen® Protocol

- Incubate beads (5 µl) and serum 25 µl (RT) 15 min.

- Wash x3 (1 min/spin) 3 min.

- Incubate with 20 µl anti-IgG-PE, 1:10 dilution (RT) 5 min.

- Wash x2 (5min/spin) 2 min.

- Total assay time 25 min.

70% time reduction!
Standard vs ROB protocol, MFI correlation

8 patient, 9 ASHI PT, 3 ABH PT sera

Class I

\[ y = 1.1103x - 66.752 \]
\[ R^2 = 0.9908 \]

Class II

\[ y = 1.1256x + 92.822 \]
\[ R^2 = 0.9845 \]
Representative Serum Reactivity
Standard vs ROB protocol

AC-463 Class I

AC-463 Class II

Liwski et al Hum. Immunol. 2017

Robert Liwski, Patricia Campbell, Adriana Colovai, Deborah Crowe, Anne Halpin, Ronald Kerman, Dong Li, John Lunz, Cathi Murphey, Peter Nickerson, Denise Pochinco, Sandra Rosen-Bronson, Olga Timofeeva, Paul Warner, Adriana Zeevi
Participating Centers

- Dalhousie University, Halifax, NS, Canada
- University of Alberta, Edmonton, AB, Canada
- Montefiore-Einstein Transplant Center, Bronx, NY
- Dialysis Clinic Inc. (DCI) Laboratory, Nashville, TN
- Baylor College of Medicine, Houston, TX
- Medstar Georgetown University Hospital, Washington, DC
- University of Pittsburgh Medical Center, Pittsburgh, PA
- Southwest Immunodiagnostics Inc. Laboratory, San Antonio, TX
- University of Manitoba, Winnipeg, MB, Canada
- Puget Sound Blood Center, Seattle, WA
Design

• 2014 ASHI PT sera
  – AC460-464

• Tested by LABScreen SAB Luminex assay
  – Standard lab method
  – ROB protocol
  – Same lot of class I and class II beads

• Result analysis:
  – MFI comparison
  – CV
  – Pearson’s correlation ($R^2$)
  – Specificity assignment
  – Pos/Neg ctrl beads (signal vs noise)
AC464 class I
Average lab MFI and CV comparison

Bead number

MFI

%CV

Bead number

Liwski et al ASHI 2014
AC460 class II
Individual lab MFI comparison

Bead number

MFI

Standard

ROB

Bead number

Liwski et al ASHI 2014
AC460 class II
Average lab MFI and CV comparison

Liwski et al ASHI 2014
Overall mean MFI correlation

Class I

$y = 0.9523x + 27.373$

$R^2 = 0.9931$

Class II

$y = 0.8709x + 36.283$

$R^2 = 0.9974$

Liwski et al ASHI 2014
Average CV
Standard vs ROB protocol

Class I

% CV

Class II

% CV

Serum

Liwski et al ASHI 2014
SORRY, WE JUST CAN’T TRUST YOU...
Conclusion

• Confirmed excellent correlation between the Standard and ROB protocols.

• Confirmed that there is no significant impact on test results when using ROB protocol.

• ROB protocol appears to improve precision of the results
Interfering Substances
“Prozone” Effect
A2 DSA
Would you transplant this patient?
A2 DSA
Would you transplant this patient?
A2 DSA

Would you transplant this patient?
Naturally occurring interference in Luminex® assays for HLA–specific antibodies: Characteristics and resolution

Andrea A. Zachary a,*, Donna P. Lucas a, Barbara Detrick b, Mary S. Leffell a

Dithiotreitol (DTT) Hypotonic dialysis ?IgM
Improved Luminex-based human leukocyte antigen-specific antibody screening using dithiothreitol-treated sera

Vasilis Kosmoliaptsis a,b, Cheryl O’Rourke a, J. Andrew Bradley b, Craig J. Taylor a,*

a Tissue Typing Laboratory, Cambridge University Hospitals NHS Foundation Trust, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom
b Department of Surgery, University of Cambridge, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom

Kosmoliaptsis et al. Human Immunology 71:45, 2010

Martina Schnaidt,¹,₄ Christof Weinstock,² Marija Jurisic,¹ Barbara Schmid-Horch,¹ Andrea Ender,³ and Dorothee Wernet¹

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**Figure A**
- MFI (Mean Fluorescence Intensity) against different sample types: native serum, 1:10 dilution, and EDTA plasma.

**Figure B**
- MFI against different sample types: native serum, 1:10 dilution, and EDTA plasma.

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Martina Schnaidt, Christof Weinstock, Marija Jurisic, Barbara Schmid-Horch, Andrea Ender, and Dorothee Wernet

C1 inhibition?
Deciphering Complement Interference in Anti–Human Leukocyte Antigen Antibody Detection With Flow Beads Assays

Jonathan Visentin,¹,² Margaux Vigata,² Sophie Daburon,² Cécile Contin-Bordes,¹,² Véronique Fremeaux-Bacchi,³ Claire Dromer,⁴ Marc-Alain Billes,⁵ Martine Neau-Cransac,⁶ Gwendaline Guidicelli,¹ and Jean-Luc Taupin¹,²,⁷
“Prozone” effect
“Prozone” effect

Low titer
Non C fixing Ab
“Prozone” effect

Low titer
Non C fixing Ab
“Prozone” effect

Low titer
Non C fixing Ab
“Prozone” effect

Low titer
Non C fixing Ab
“Prozone” effect

High titer
C fixing Ab

SAB
HLA-A2
“Prozone” effect

High titer
C fixing Ab
“Prozone” effect

High titer
C fixing Ab
C1q binds

SAB HLA-A2
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
“Prozone” effect

High titer C fixing Ab
C1q binds C1r & C1s recruited
C4 converted to C4b

Ca$^{2+}$
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex

Ca^{2+}
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a

Ca$^{2+}$

SAB
HLA-A2
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b

Ca^{2+}
“Prozone” effect

High titer C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
C3b binds HLA-Ab complex and C4b2a (C5 convertase)
“Prozone” effect

High titer C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
C3b binds HLA-Ab complex and C4b2a (C5 convertase)
“Prozone” effect

High titer
C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
C3b binds HLA-Ab complex and C4b2a (C5 convertase)

Binding of anti-IgG-PE is blocked
HLA antibody not detected
“Prozone” effect

High titer C fixing Ab
C1q binds
C1r & C1s recruited
C4 converted to C4b
C4b binds HLA-Ab complex
C2 converted to C2a
C2a binds C4b (C3 convertase)
C3 converted to C3b
C3b binds HLA-Ab complex and C4b2a (C5 convertase)

Binding of anti-IgG-PE is blocked
HLA antibody not detected

Solutions:
Heat treatment (56°C), destroys C1q and other C
Serum dilution, dilutes out complement
DTT, breaks C1q
EDTA, chelates Ca^{2+}
“Prozone” effect

EDTA $\times$ $Ca^{2+}$

SAB
HLA-A2
“Prozone” effect

EDTA $\times^{2+}$

SAB
HLA-A2
“Prozone” effect

EDTA $\text{Ca}^{2+}$
IgG SAB neat

IgG SAB 1:10

IgG SAB EDTA

C1q

“prozone” effect
Comprehensive assessment for serum treatment for single antigen test for detection of HLA antibodies

Xiaohai Zhang*, Nancy L. Reinsmoen

HLA and Immunogenetics Laboratory, Comprehensive Transplant Center, Cedars-Sinai Health System, 8723 Alden Drive, SSB 197, Los Angeles, CA 90048, United States

Fig. 1. MFI of each bead in single antigen testing on 7 sera treated with EDTA (A), heat (B), or DTT (C) was plotted against MFI in untreated sera in scatter plots. All these treatments significantly increased MFI of many beads which are positioned above the diagonal line. The dashed lines represent the cutoff for single antigen testing on untreated sera. Antibodies against many HLA antigens that were negative (on the left side of the lines) in untreated sera became strong positive after treatments.
Comprehensive assessment for serum treatment for single antigen test for detection of HLA antibodies

Xiaohai Zhang*, Nancy L. Reinsmoen

HLA and Immunogenetics Laboratory, Comprehensive Transplant Center, Cedars-Sinai Health System, 8723 Alton Drive, SSB 197, Los Angeles, CA 90048, United States

Fig. 2. Comparison of MFI in single antigen testing between different treatments. (A) MFI of DTT treated sera was plotted against EDTA treated sera. (B) MFI of DTT treated sera was plotted against heat inactivated sera. (C) MFI of heat inactivated sera was plotted against EDTA treated sera. The lines inside the graph indicate the cutoff of MFI 2500. MFI of heat inactivated sera was highly correlated with that of EDTA treated sera as indicated by a correlation coefficient (r) of 0.99.
Comprehensive assessment for serum treatment for single antigen test for detection of HLA antibodies

Xiaohai Zhang*, Nancy L. Reinsmoen

HLA and Immunogenetics Laboratory, Comprehensive Transplant Center, Cedars-Sinai Health System, 8723 Alden Drive, SSB 197, Los Angeles, CA 90048, United States

Fig. 3. Antibodies which were revealed by dilution could also be detected after DTT, heat, or EDTA treatment. (A) Comparison of MFI among dilution, EDTA, heat, and DTT treatment. (B) MFI of each antigen in single antigen testing on 1:8 diluted, EDTA treated sera against MFI in EDTA treated, undiluted sera in a scatter plot. The lines inside the graph indicates the cutoff of MFI 2500. The upper right quadrant represents antibodies positive in both undiluted and 1:8 diluted sera. Antibodies that were not detected in undiluted sera but became positive after 1:8 dilution would be in the upper left quadrant if there were such antibodies.
Treatment of sera with EDTA

• Simple and effective solution to prevent “prozone”.

• Anecdotal evidence that the procedure is not effective.

• The protocol for serum treatment with EDTA published by Schnaidt et. al. is very ambiguous and impossible to follow.

  • “5μL EDTA solution (6%) + 95μL of serum”
Treatment of sera with EDTA protocol

• Schnaidt et al: “5μL EDTA solution (6%) + 95μL of serum”

• Does not indicate which EDTA salt is used:
  • Disodium EDTA, MW = 292.24
  • Dipotassium EDTA, MW = 368.42
  • Dipotassium EDTA dihydrate, MW = 404.47

• Does not indicate if solid or solution used.

• Does not indicate whether 6% means v/v or w/v
Divalent Metal Ion, Reference Ranges

<table>
<thead>
<tr>
<th>Divalent Metal Ions in Blood</th>
<th>Normal Range (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (Total)</td>
<td>2.18-2.58</td>
</tr>
<tr>
<td>Calcium (Ionized)</td>
<td>1.05-1.3</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.75-0.95</td>
</tr>
<tr>
<td>Iron</td>
<td>0.011-0.032</td>
</tr>
<tr>
<td>Copper</td>
<td>0.011-0.025</td>
</tr>
</tbody>
</table>

- EDTA forms a 1:1 complex with divalent metal ions
- Required amount of EDTA should “mop up” all divalent metal ions in serum (ionized calcium and magnesium), > 1.8 - 2.5 mmol/L.
Experimental design

• Six “prozone” positive sera (3 class I and 3 class II) were selected based on dilution studies.

• Sera were treated with different concentrations of EDTA or PBS control.
  • Disodium EDTA, 0.5M stock solution, Sigma-Aldrich
  • Final EDTA concentrations: 2mM, 3mM and 6mM

• Tested sera using LABScreen Single antigen bead assay
Class I HLA, representative serum

PBS

2mM EDTA

3mM EDTA

6mM EDTA
Class II HLA, representative serum

- PBS
- 2mM EDTA
- 3mM EDTA
- 6mM EDTA
Class I HLA

“Prozone” positive specificities

![Bar graph showing specificities of Class I HLA with EDTA](image)

- Specificities (sorted in order of MFI for no EDTA)
- EDTA
  - 6mM
  - 3mM
  - 2mM
  - no EDTA
Class II HLA

“Prozone” positive specificities

MFI

Specificities

(sorted in order of MFI for no EDTA)

EDTA

- 6mM
- 3mM
- 2mM
- no EDTA
Conclusions

• EDTA treatment of serum is an effective way to eliminate “prozone” effect.

• Concentration of EDTA is important when treating serum.

• Minimal dose of EDTA needed to prevent “prozone” is ≥ 3mM.

• This concentration of EDTA is necessary to chelate all divalent metal ions in blood.
Questions

• How common is prozone?

• Propensity to prozone?

• Degree of prozone?

• Mechanism of prozone and relative contribution of IgM vs C3d?

• Loci affected?
Prevalence, distribution and amplitude of the complement interference phenomenon in single antigen flow beads assays

G. Guidicelli¹ | J. Visentin¹,²,³ | N. Franchini¹ | C. Borg¹ | P. Merville²,³,⁴ | L. Couzi²,³,⁴ | J.-L. Taupin¹,²,³

- Studied 129 class I and 85 class II patient sera
- Complement interference is common
  - 30% class I HLA sera
  - 46% class II HLA sera
The ABCs (DRDQDPs) of the prozone effect in single antigen bead HLA antibody testing: Lessons from our highly sensitized patients

Anna L. Greenshields, Robert S. Liwski*

Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada

- 30 highly sensitized patients (cPRA ≥ 95%) on active renal waitlist
- 18 female (60%), 12 male (40%)

Female patient sensitization history:
- 6 pregnancy (+/- transfusion 50%)
- 6 transplant (+/- transfusion 50%)
- 6 transplant + pregnancy (+/- transfusion 67%)

Male patient sensitization history:
- 12 transplant (+/- transfusion 67%)
The ABCs (DRDQDPs) of the prozone effect in single antigen bead HLA antibody testing: Lessons from our highly sensitized patients

Anna L. Greenshields, Robert S. Liwski*

Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada

Highly sensitized patients (n=30): cPRA ≥ 95%
The ABCs (DRDQDPs) of the prozone effect in single antigen bead HLA antibody testing: Lessons from our highly sensitized patients

Anna L. Greenshields, Robert S. Liwski*

Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada

- LABScreen SAB assay
  - IgG +/- EDTA to identify prozone, +/- dilution
  - C3d, IgM
- Total of 6049 specificities (3,104 class; 2,945 class II) analysed
  - IgG negative: n = 3700 (61%)
  - IgG positive: n = 2349 (39%)
    - No prozone (ΔMFI EDTA < 3K): n = 1690 (72%)
    - Prozone (ΔMFI EDTA > 3K): n = 659 (28%)
A) IgG –ve (n=3,518) EDTA IgG-PE MFI < 1,000

EDTA IgG-PE
MFI

IgG-PE
MFI

C3d-PE
MFI

C3d +ve = 0.17%

B) IgG +ve, prozone –ve (n=1,726) IgG-PE ΔMFI < 3,000

C3d +ve = 10.6%

MFI = 3,152+/−1,848
A) IgG -ve (n=3,518) EDTA IgG-PE MFI < 1,000

EDTA IgG-PE
MFI

IgG-PE
MFI

C3d-PE
MFI

C3d +ve = 0.17%
MFI = 3,152+/−1,848

IgM-PE
MFI

IgM +ve = 4.7%
MFI = 2,406+/−1,564.9

B) IgG +ve, prozone -ve (n=1,726) IgG-PE ΔMFI < 3,000

C3d +ve = 10.6%
MFI = 2,327+/−1,796.9
Mild prozone (n=88)
IgG-PE ΔMFI=3,000-4,999

Moderate prozone (n=131)
IgG-PE ΔMFI=5,000-9,999

Marked prozone (n=297)
IgG-PE ΔMFI≥10,000

C3d+ = 87.5%
MFI=5,878+/−2,462

IgM+ = 13.6%
MFI = 2,847+/−1,515

C3d+ = 100%
MFI=9,593+/−1,972

IgM+ = 29.8%
MFI = 2,898+/−1,330

C3d+ = 100%
MFI=15,572+/−2,945

IgM+ = 64.0%
MFI = 4,794+/−2,965

Greenshields and Liwski Hum. Immunol. 2019
A) Mild prozone (n=88)
IgG-PE ΔMFI=3,000-4,999

B) Moderate prozone (n=131)
IgG-PE ΔMFI=5,000-9,999

C) Marked prozone (n=297)
IgG-PE ΔMFI≥10,000

EDTA IgG-PE

MFI

MFI

MFI

IgG-PE

C3d-PE

C3d +ve = 87.5%
MFI=5,878+-2,462

C3d +ve = 100%
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Greenshields and Liwski Hum. Immunol. 2019
A) Mild prozone (n=88)
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IgG-PE ΔMFI=5,000-9,999

C) Marked prozone (n=297)
IgG-PE ΔMFI≥10,000

EDTA IgG-PE

IgG-PE

C3d-PE

IgM-PE

Greenshields and Liwski Hum. Immunol. 2019
Degree of prozone correlates with C3d deposition

C3d MFI ≥ 4,000 predicts prozone
sensitivity = 95.2%
specificity = 97.2%

Greenshields and Liwski Hum. Immunol. 2019
Dilution did not reveal additional mechanisms of “prozone”.

A) EDTA undiluted MFI vs EDTA + 1:10 dilution MFI

0 5000 10000 15000 20000 25000 30000
0 5000 10000 15000 20000 25000 30000
Frequency and severity of prozone in HSP

Frequency of prozone:
- None: 56.25%
- Class I only: 15.625%
- Class II only: 18.75%
- Both: 9.375%

Severity of prozone:
- None: 71.875%
- Marked: 15.625%
- Moderate: 6.25%
- Mild: 3.125%
- Borderline: 3.125%
Frequency of prozone by HLA class and locus

A) Prozone by HLA class
- 70% Class I
- 30% Class II

B) Prozone by locus, Class I
- 62% A
- 6% B
- 32% C

C) Prozone by locus, Class II
- 18% DR
- 14% DQ
- 68% DP

D) Frequency of prozone by HLA class and locus

<table>
<thead>
<tr>
<th>HLA Locus</th>
<th>Prozone pos/total pos (%)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>35</td>
</tr>
<tr>
<td>B</td>
<td>30</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
</tr>
<tr>
<td>DR</td>
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<tr>
<td>DQ</td>
<td>25</td>
</tr>
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<td>DP</td>
<td>10</td>
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</tbody>
</table>
Degree of prozone by sensitizing event

![Bar chart showing the percentage of patients with different degrees of prozone by sensitization event. The x-axis represents the type of sensitization event: Pregnancy, Transplant, and All. The y-axis represents the percentage of patients. The chart categorizes prozone as None, Mild, and Mod/Marked.]
How can we identify sera that exhibit prozone?

Positive control bead?  
Negative control bead?
Positive control bead reactivity

MFI

Untreated  EDTA

Positive control (Bead #2)
Negative control bead reactivity

Negative Control (Bead #1)

MFI

Untreated    EDTA
Summary

• The “prozone” effect is very common among the highly sensitized patients (84%; 75% marked).

• Main risk factor: history of previous transplant

• Class I more commonly affected than class II
  • Most commonly affected loci: HLA-A, B and DQ

• Due to complement mediated interference.
  • Contribution from IgM?

• Degree of interference is proportional to the amount of complement deposition

• EDTA is effective at minimizing complement mediated interference
Denatured HLA epitopes
Patient Case

Would you transplant this patient?

<table>
<thead>
<tr>
<th>Patient</th>
<th>DD kidney</th>
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<tbody>
<tr>
<td>A</td>
<td>24 29</td>
</tr>
<tr>
<td>B</td>
<td>44:03 48</td>
</tr>
<tr>
<td>C</td>
<td>08 16</td>
</tr>
<tr>
<td>DRB1</td>
<td>07 12</td>
</tr>
<tr>
<td>DRB3/4/5</td>
<td>52 53</td>
</tr>
<tr>
<td>DQB1</td>
<td>02:02 03:01</td>
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<tr>
<td>DQA1</td>
<td>02:01 05</td>
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<td>01 02</td>
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<td>44:02 57</td>
</tr>
<tr>
<td></td>
<td>05 06</td>
</tr>
<tr>
<td></td>
<td>01 07</td>
</tr>
<tr>
<td></td>
<td>Blank 53Null</td>
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<tr>
<td></td>
<td>03:03 05</td>
</tr>
<tr>
<td></td>
<td>01 02:01</td>
</tr>
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</table>

Previous cardiac transplant

Unacceptable antigens
B8, B37, B41, B42, B44(44:02), B45, B82
Allo X-match

FCXM results

T cell

B cell

Neg control

Patient

Pos control

<table>
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<tr>
<th></th>
<th>T cell</th>
<th>B cell</th>
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<tr>
<td>Neg Control</td>
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<td>229</td>
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<tr>
<td>Patient</td>
<td>166</td>
<td>201</td>
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<tr>
<td>Pos Control</td>
<td>493</td>
<td>500</td>
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</tbody>
</table>
### Unacceptable antigens

B8, B37, B41, B42, **B44(44:02)**, B45, B82
Epitopes of human leukocyte antigen class I antibodies found in sera of normal healthy males and cord blood

Nadim El-Awar a,*, Paul I. Terasaki b, Anh Nguyen a, Nori Sasaki a, Luis E. Morales-Buenrostro c, Hiroh Saji d, Etsuko Maruya d, Francesca Poli e

Table 1
Fifty-eight mostly cryptic epitopes located on dissociated class I HLA antigen (heavy chain)

<table>
<thead>
<tr>
<th>Dissociated antigen(s) with distinct epitope*</th>
<th>Epitope no. assigned</th>
<th>Possible epitope site</th>
<th>Epitope description</th>
<th>Ag form</th>
<th>MX</th>
<th>jf</th>
<th>CF</th>
<th>FI</th>
<th>Total</th>
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<tbody>
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<td>A2</td>
<td>5001</td>
<td>(74D)</td>
<td>C</td>
<td>D</td>
<td>4</td>
<td>1</td>
<td>5</td>
<td></td>
<td></td>
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<tr>
<td>A2, A12, A24</td>
<td>5002</td>
<td>(6,5) × (70D)</td>
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<td>D</td>
<td>3</td>
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<tr>
<td>A25, A26, A21, A30, A24, A66, A602, A682, A69</td>
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<td>(31A)</td>
<td>C</td>
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<td>4</td>
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<td>B2705, B2708</td>
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<tr>
<td>B27B2, B2737</td>
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<td>1</td>
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<td>B27B2</td>
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<td>B37</td>
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</table>

Positive virtual crossmatch with negative flow crossmatch results in two cases

Eapen K. Jacob, Steve R. De Goey, Manish J. Gandhi *

Division of Transfusion Medicine, Department of Laboratory Medicine and Pathology, Mayo Clinic College of Medicine, Rochester, MN 55905, United States
Acid treatment denatures class I HLA antigens
Acid treatment, patient serum

Untreated

Acid Tx

↑ A31 & A33
Denatured epitope, 73I
Table 1
Fifty-eight mostly cryptic epitopes located on dissociated class I HLA antigen (heavy chains)

<table>
<thead>
<tr>
<th>Dissociated antigen(s) with distinct epitope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Epitope no. assigned&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Possible epitope site&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Epitope description&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ag. form&lt;sup&gt;e&lt;/sup&gt;</th>
<th>MX&lt;sup&gt;f&lt;/sup&gt;</th>
<th>JE&lt;sup&gt;g&lt;/sup&gt;</th>
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<td>A23,A24</td>
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<td>B27,B37</td>
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<td>B37</td>
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<td>11</td>
<td>1</td>
</tr>
<tr>
<td>B37,B47</td>
<td>5017</td>
<td>(70N)+(77D)</td>
<td>C</td>
<td>D</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B4402</td>
<td>5018</td>
<td>(77N)+(156D)</td>
<td>C</td>
<td>D</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>B5501</td>
<td>5019</td>
<td>(97T)+(116L)+(152E)</td>
<td>C</td>
<td>D</td>
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</tr>
<tr>
<td>B57,B58,B63</td>
<td>5020</td>
<td>(70S)</td>
<td>C</td>
<td>D</td>
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<td></td>
</tr>
<tr>
<td>B65</td>
<td>5021</td>
<td>(11A)+(97W)</td>
<td>C</td>
<td>D</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B67</td>
<td>5022</td>
<td>(70Q)+(116F)</td>
<td>C</td>
<td>D</td>
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</tr>
<tr>
<td>B7,B48,B60,B81</td>
<td>5023</td>
<td>(178K)</td>
<td>D</td>
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<tr>
<td>B7,B42,B54,B55,B56,B67, B81, B82</td>
<td>5024</td>
<td>(66I)+(70Q)</td>
<td>C</td>
<td>D</td>
<td>8</td>
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</tr>
<tr>
<td>B72</td>
<td>5025</td>
<td>(45E)+(77S)+(116S)</td>
<td>C</td>
<td>D</td>
<td>2</td>
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</tr>
<tr>
<td>B75</td>
<td>5026</td>
<td>(67S)+(77S)+(95I)</td>
<td>C</td>
<td>D</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>5027</td>
<td>(9D)</td>
<td>D</td>
<td></td>
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<td></td>
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<tr>
<td>B8,B37,B42,B82</td>
<td>5028</td>
<td>(24S)+(156D)</td>
<td>C</td>
<td>D</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>B8,B42,B82</td>
<td>5029</td>
<td>(45E)+(156D)</td>
<td>C</td>
<td>D</td>
<td>2</td>
<td>1</td>
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<tr>
<td>B8,B42,B37,B41,B4102, B44, B45,B82</td>
<td>5030</td>
<td>(156D)</td>
<td>C</td>
<td>D</td>
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<tr>
<td>B82</td>
<td>5031</td>
<td>(24S)+(99F)</td>
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<td>13</td>
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<tr>
<td>Cw1</td>
<td>5032</td>
<td>(6K)</td>
<td>C</td>
<td>D</td>
<td>1</td>
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<tr>
<td>Cw2</td>
<td>5033</td>
<td>(211T)</td>
<td>C</td>
<td>D</td>
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<tr>
<td>Cw1502</td>
<td>5034</td>
<td>(1C)+(116L)</td>
<td>C</td>
<td>D</td>
<td>4</td>
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</tr>
<tr>
<td>Cw16</td>
<td>5035</td>
<td>(116S)+(156Q)</td>
<td>C</td>
<td>D</td>
<td>4</td>
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<tr>
<td>Cw17</td>
<td>5036</td>
<td>(116F)+(143S)</td>
<td>C</td>
<td>D</td>
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<tr>
<td>Cw4,Cw6,Cw17,Cw18</td>
<td>5037</td>
<td>(73A)+(77N)</td>
<td>C</td>
<td>D</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cw6</td>
<td>5038</td>
<td>(9D)+(97W)</td>
<td>C</td>
<td>D</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Cw7</td>
<td>5039</td>
<td>(66K)+(99S)</td>
<td>C</td>
<td>D</td>
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</tr>
</tbody>
</table>
Studied a cohort of 323 renal wait list patients
- 40% with class I HLA cPRA of > 85%
- 12% with class I HLA cPRA = 0
- 91% experienced at least one sensitizing event

Sera tested by standard SAB, acid denatured SAB and iBead SAB for class I HLA antibodies

Correlation with FCXM

Impact on cPRA
83% FCXM positive

16% FCXM positive

Visentin et al. Transplantation 98:738 2014
The significance of pretransplant donor-specific antibodies reactive with intact or denatured human leucocyte antigen in kidney transplantation

H. G. Otten, M. C. Verhaar, H. P. E. Borst, M. van Eck, W. G. J. van Ginkel, R. J. Hené and A. D. van Zuilen

- Retrospective study of 837 renal transplant recipient transplanted with negative CDC T/B XM

- Pre-transplant sera were retrospectively tested by standard SAB, acid denatured SAB and iBead SAB
  - DSA classified into native vs denatured epitope specific

- 156 patients were found to have class I HLA DSA by standard SAB, 11% had class I DSA directed against denatured epitopes only.

- Correlation with graft survival.


(a)

Denatured epitope

No DSA

Native epitope (iBeads)

Graft survival (%)

Time (years)

Class-I DSA (n = 156)
no class-I DSA (n = 681)
Class-I DSA iBeads (n = 120)
no class-I DSA iBeads (n = 36)
DSA exclusively against denatured class-I (n = 20)

P = 0.007
P = 0.002
Antibodies against denatured HLA epitopes

• Clinical significance is questionable

• Prevalence
  • 20-40% of patients (Visentin et al.), 11% of DSA (Otten et al.)
  • Significant effect on cPRA and allocation (Visentin et al.)

• Solutions:
  • Acid treatment (class I)
  • iBeads (class I), no longer available
  • Epitope analysis, pattern recognition.
  • Correlation with FCXM +/- absorption elution studies
Complement-Binding Anti-HLA Antibodies and Kidney-Allograft Survival

Probability of Graft Survival

Years after Transplantation

P < 0.001 by log-rank test

Loupy et al. *NEJM*. 2013
Complement-Binding Anti-HLA Antibodies and Kidney-Allograft Survival

Probability of Graft Survival

- DSA-
- DSA+, C1q-
- DSA+, C1q+

Years after Transplantation

P < 0.001 by log-rank test

Graft survival Probability

logrank p < 0.0001

Time post transplantation (years)

< 6,000

> 6,000

Loupy et al. NEJM. 2013
Complement-Binding Anti-HLA Antibodies and Kidney-Allograft Survival

Prozone? < 6,000 > 6,000

P < 0.001 by log-rank test

Loup et al. NEJM. 2013
Complement-Binding Anti-HLA Antibodies and Kidney-Allograft Survival

**Probability of Graft Survival**

- DSA- (n=700)
- DSA+/C1q- | MFI < 6000 (n=215)
- DSA+/C1q- | MFI >=6000 (n=24)
- DSA+/C1q+ | MFI < 6000 (n=27)
- DSA+/C1q+ | MFI >=6000 (n=50)

- P<0.001 by log-rank test

**Graft survival Probability**

- Time post transplantation (years)

Logrank p < 0.0001

Prozone? < 6,000

Denatured? > 6,000

Loupy et al. *NEJM*. 2013
The disappointing contribution of anti-human leukocyte antigen donor-specific antibodies characteristics for predicting allograft loss

Maxime Courant\textsuperscript{1,*}, Jonathan Visentin\textsuperscript{2,3,*}, Gabriel Linares\textsuperscript{2}, Valérie Dubois\textsuperscript{4}, Sébastien Lepreux\textsuperscript{5,6}, Gwendaline Guidicelli\textsuperscript{4}, Olivier Thaunat\textsuperscript{7,8}, Pierre Merville\textsuperscript{1,3}, Lionel Couzi\textsuperscript{1,3,*} and Jean-Luc Taupin\textsuperscript{2,3,9,*}
Requirements for successful and safe virtual crossmatching

- Accurate patient sensitization history, including immunosuppression
- HLA typing
  - All loci
  - SABR resolution
- Up to date SAB HLA antibody information
  - On call SAB testing and vXM
- Reproducible SAB and FCXM
- Serum treatment with EDTA/DTT/Heat to eliminate the prozone effect
- HLA antibody assignment
  - Epitope analysis
  - Denatured epitopes/non-specific reactivity
Thank you!

Questions?