Panel Reactive Antibodies and Crossmatch by Flow Cytometry
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Renal transplantation
In spite of the enormous progress in the techniques
• Surgery
• Graft preservation
A main limitation for the successful result of a transplantation especially renal and to a lesser extent of other organs remains
the recipient’s immune response to the donor cells/tissues
↓ rejection
↓ Graft loss

Variable factors contribute to progressive malfunction

Loss of the allograft
(chronic rejection remains a challenge)

antibody-mediated rejection - AMR
consists the dominant cause of graft loss

Antibody - mediated rejection (AMR)

Antibodies against
• Major Histocompatibility Systems (polymorphic)
  - RBC antigens (mainly ABO system)
  - MHC - Major Histocompatibility Complex antigens known in humans as
    - HLA - Human Leukocyte Antigens
• Minor Histocompatibility Systems
  (no significance in clinical practice)
  - Endothelial cell antigens
    (most non polymorphic)
  Antiendothelial Cell Antibodies - AECAs

AMR diagnosis confirmation (revised Banff criteria)
demands
• histologic lesions in renal biopsy (acute tissue destruction)
• recent interaction of abs with vascular endothelium
• detection of DSA (Donor-Specific Antibodies) in the serum
  - HLA or non-HLA
  - preformed or de novo
Significance of ABO compatibility in renal transplantation

People without A or B antigens have anti-A and anti-B antibodies in serum.

Transplantation with major ABO incompatibility leads to hyperacute rejection.

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Trials of ABO incompatible renal transplantations:

- A2O (due to A2 low antigenicity)
- Transplantation from live ABO incompatible donor
- Plasmapheresis
- B cells depletion (in place of splenectomy)
- Immunosuppressive treatment

Trials of ABO incompatible renal transplantations after:

- Plasmapheresis
- B cells depletion (in place of splenectomy)
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Transfusion rules are followed.

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MHC region

- Situated in the short arm of chromosome 6
- It covers PABP locus pair (PAI)
- Genes are classified into class I, II, III

HLA antigens

- Glycoproteins
  - Cell membrane
- Classified
  - Class I
    - HLA-A, -B, -Cw
  - Class II
    - HLA-DR, -DQ, -DP

Sensitization: generation in the recipient of abs against allogeneic HLA antigens

- Blood transfusions (multiple)
- Pregnancies (women with many children)
- HLA incompatible allograft rejection
- Venous or arterial allograft
- Infections
- Trauma
- Mucocutaneous
Sensitization against multiple HLA antigens in one transplantation.

Due to shared epitopes in different HLA antigens.

The role of transfusions in sensitization

- Transfusions increase sensitization risk
- 20% sensitization or panel reactive antibodies (PRA) increase
- If it is possible transfusions should be avoided in all candidate renal recipients

anti-HLA antibodies detection methods

Panel-reactive antibodies (PRA)
Donor-specific antibodies (DSA)

1. Using cells
   - Complement-dependent Cytotoxicity (CDC)
   - Flow cytometry (Flow PRA)
2. Solid phase
   - Enzyme-linked Immunosorbent Assay (ELISA)
   - Bead Array Assay (Luminex) - single antigen beads (SAB)

PRA using cells and CDC

an example

Hyper-sensitized patients: with PRA > 70% (class I or/and class II)

The Concept of FlowPRA™ Test

FlowPRA Class I Specific Tests

Pioneer Multiplexed Bead Assay
**FlowPRA™ II Specific Tests**

- **Bead Array Assay (SAB) results**
  - Mean Fluorescence Intensity (MFI)
  - It depends on the quantity and the strength of the Ab.

**Bead Array Assay (Luminex)**

**Main advantages**
- Identification of Abs against specific HLA antigens (one antigen per bead) → Virtual Xmatch

**Disadvantages**
- Very sensitive method → false positive results
- It doesn't discriminate between Abs activating or not complement
- It is not clear whether these Abs have any clinical significance
- The method is not standardized

** Specific crossmatch - XM **

**Advantages**
- The only method detecting only Abs complement activating (greater clinical significance)
  - The only functional method
  - It imitates in vivo reality

**Disadvantages**
- It does not detect low titer Abs
  - False negative results
- False positive results in the presence of autoAbs, non-HLA Abs, high background (especially regarding B cells)
- Less sensitive than FC-XM
- It does not detect clinically significant Abs not activating C

**CDC-XM**

- The only method detecting only Abs complement activating (greater clinical significance)
- The only functional method
- It imitates in vivo reality
Clinical significance of preformed DSA abs

It is generally accepted that the presence of preformed HLA class I or II DSA (IgG) is correlated with a higher rejection risk.

- HLA-A, B, (Cw), DR DSA: Their role has been documented years ago
- HLA-DQ, DP DSA (detected with Luminex & FC)
- Correlated with acute/intermediate rejection risk
- If their presence combined with IgG CDC-XM (+) — high risk
- HLA-DP DSA: They will possibly lead to acute or chronic rejection (although weakly expressed on renal endothelial cells)
- Complement DSA subclass determination (C1q assay): It is suggested as the method determining whether the detected Abs are involved in rejection pathogenesis

DSA (+) double risk of AMR
CDC-XM (+) ×
FC-XM (+) × 76% greater graft loss (meta-analysis)

Sensitization (anti-HLA Abs development) clinical significance in renal transplantation

Annals of the New York Academy of Sciences 2013, 1283: 30-42

- CDC-XM (+): Absolute contraindication for transplantation
- FC-XM (+): Relative
- DSA detected only with solid-phase methods (Luminex)
- ↑ risk for graft rejection and graft loss
- Generally accepted cutoffs have not been determined and they vary between centers
- Stronger immunosuppression and stricter follow-up

- Clinical significance not clear
- They are not generally harmful for the graft
- Positive IgM FC-XM (T cells): could be a risk factor for graft rejection (not performed in most centers)
endothelial XM (XM-ONE)

detecting abs on endothelial cells

Do Abs detected by XM-ONE link on the graft differentiated endothelial cells??

It is very important to follow up the development of de novo anti-HLA abs. Chronic rejection may be predicted by the development of HLA-specific antibody following transplant (Terasaki and Ozawa, 2004)

Flow cytometry crossmatch
FCXM

The procedure
- Sample preparation
- Donor PBMCs

Donor PBMCs
- Overlay WB (preferably in heparin) on Ficoll
- Collect PBMCs
- Wash and prepare a sample of 3*10^6/ml concentration

history
- Introduced by Garavoy et al. (1983)
- Cook et al., 1987; Lazda et al., 1988; Mahoney et al., 1990; Ogura et al., 1993
- 2 color FC (separate tubes for T and B cells)
### Xmatch incubation

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<th>T+ positive control</th>
<th>T+ recipient serum</th>
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<tr>
<td>Negative control</td>
<td>50 μl cells + 50 μl negative serum</td>
<td>50 μl cells + 50 μl positive serum</td>
<td>50 μl cells + 50 μl recipient serum</td>
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<tr>
<td>B+ control</td>
<td>50 μl cells + 50 μl negative serum</td>
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Negative: All serum pool or commercial preparation e.g. NIBSC
Positive: multisensitized patient serum or commercial preparation

### Anti-human IgG incubation

- Anti-human (goat? Sheep?) IgG-FITC (Fab)2 Fc specific
- CD3 for T cells
- CD19 or CD20 for B cells
- 2 or 3 colour
- Washing steps

### 3-Color Flow Cytometric Crossmatch

- Robert A. Bray et al 2011
- THREE-COLOR: Anti-T and Anti-B Cell CROSS-MATCH

#### Positive T-Cell XAM

- CD3 PE Control
- CD3 PE Test

#### Positive B-Cell (and T-Cell) XAM

- CD19 APC Control
- CD19 APC Test

**T negative**

**T positive**
T donor - recipient

B negative

B positive

B donor - recipient

Ratio MFI
- T cells
  - 0.38/0.25 = 1.52
- B cells
  - 0.77/0.55 = 1.4

UKNEQAS FCXM
Our results

The report

Methods for the determination of the cut-off value for the positive

- Mean channel shift (MCS) after subtracting mean channel fluorescence (MCF) of the negative control serum from MCF of the recipient serum
  - T CXM boundary: if \( \leq 40 \) or \( \geq 80 \)
  - 50% T CXM boundary: if \( \geq 100 \) or \( \geq 200 \) (Potef et al. 2007)
- Ratio MESF recipient / MESF negative
  - T CXM: if ratio \( \leq 1.4 \)
  - 50% T CXM: if ratio \( > 1.2 \)

Flow cytometric crossmatch

Behaviour of polyclonal sheep anti-Hu IgG FITC

Correct behaviour

Frequent occurrence of uncorrect behaviour of polyclonal antisera

Many more methods for the determination of the cut-off value for the positive

- Median difference
- Ratio of median fluorescence of test to negative
- Linear channel shift
- Test of negative control MESF ratio
- Fluorescence index
- 8000 mean negative control

Clinical decisions

If negative ok to proceed?

- Most yes
  1. We also use the term weakly positive if the test/neg MESF value is between 1.2 and 1.5.
  2. As no other clinical data is available to review with the flow crossmatch results then pos/neg along with equivocal is adequate.
  3. The option "Equivocal result" is interesting.
Clinical decisions
If negative ok to proceed?
- If no
1. “Ok to proceed” - if positive discussion with clinician considering patient history, sensitization and clinical need
2. Immunological risk. Would need to know HLA mismatch and antibody levels
3. An equivocal range is a good idea
4. The positive result should be explained in relation to the presence of antibodies.
5. Low positive would also be useful.
6. The result seems to be equivocal because they are close to the positive threshold. In general, our clinical rules consist of investigating the whole historical medical patient file.
7. A high proportion of samples sent are marginally positive. In practice such results are evaluated after consideration of other laboratory and clinical data.
8. It would be useful to include a “Weakly reactive/Borderline” result option. In clinical terms, there is a big difference between “definitely no reaction” and “probably something.”
9. Sometimes results are indeterminate or borderline. Insufficient samples are sent so they cannot be tested in duplicate as the patient samples are.

Potential reactivity patterns
- T cell
  - Negative
  - Positive
- B cell
  - Negative
  - Positive
- Interpretation
  - No HLA antibody or very low titer
  - Predominantly Class II antibody
  - Weak Class I or II antibody
  - Strong Class I antibody or a combination of Class I and Class II
  - Likely non-HLA reactivity
  - Assumes that HLA specificity has been confirmed via alternative solid phase testing methods

Robert A. Bray

Clinical significance of FCXM
Upon multivariate adjustment with Cox regression, T FCXM+/CDCXM- deceased donor transplantation was associated with 51% higher adjusted relative risk of 1-year graft loss than FCXM-/CDCXM-.

Relative risks were more marked at 1 year for the T groups but stronger in the 1-5 year interval for the B groups.

R.J. Graff et al (2010)

Effect of blood group incompatibility on T FCXM.

M. Lindemann et al
Cytometry 2016

CDC XM (-) FCXM (+)
- Perhaps due to low levels of abs
- Very significant for 2nd transplantation and for sensitized patients
Due possibly to memory clones, which give rise to new abs.

Scornik et al. 1992

Clinical significance of B(only)-FCXM+
- Specificity of anti-donor antibodies causing the B-FCXM
- Detection of noncomplement-fixing antibodies
- Level of expression of HLA class II antigens on kidney endothelial cells
- Diagnosis and immunological status of patient prior to kidney transplantation
- Immunosuppression protocol
- Level of antibody found in the B-FCXM assay
- Sensitivity of the flow cytometric instrument

Julie C. Delgado, David D. Eckels (2008)
Specificity of DSA giving rise to B (only)+ FCXM

- Antibody specificity
- Autoantibody
- Anti-HLA class I antibody
- Anti-HLA DR antibody
- Anti-HLA DQ, DP antibody
- Antibodies against minor antigens (e.g., MICA)
- IgG1, IgG3 subclass antibody
- IgG2, IgG4 subclass antibody

Risk category: High, Low

Julio C. Delgado, David D. Eckels (2008)

More advantages of FCXM

- T and B cells XM
- Different classes of antibodies (IgM, IgA)
- Binding assay reduces the chances of technical failure and inconsistencies
- Instrumental in helping promote a new approach to living donor transplantation, namely “kidney paired donor” exchanges (Rees M., et al 2009)

Drawbacks of FCXM

- Nonspecific immunoglobulin binding to Fc receptors can be mistaken as a positive result.
- Non-HLA antibodies (e.g., autoantibody) may also bind to the cells producing a positive result.
- Monoclonal anti-lymphocyte antibodies such as Thymoglobulin, Rituxan, and Campath (used for immunosuppressive therapy) can also interfere with the FCXM and produce a “false” positive result.
- High dose IVIG, used in some desensitization protocols, can also interfere with the FCXM.

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