Flow-cytometry methods to investigate anti-platelet immunity

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Immunity against platelets

- Autoimmune thrombocytopenia
- Foetal / neonatal alloimmune thrombocytopenia (FNAIT)
- Platelet transfusion refractoriness
How do we measure the effect of platelet transfusions?

- Bleedings stop.
- Platelet counts in the blood increase.
  
  Standardized measurement is the Corrected Count Increment (CCI) calculated from:
  
  - Platelet increment (PI): difference in platelet count before and after transfusion
  - Body surface area (BSA)
  - Platelet dose (PD)

\[
CCI = PI \times BSA \times PD^{-1}
\]

A CCI of \(\geq 7.5 \times 10^9\) m\(^2\)/L is considered an acceptable response.
Platelet transfusion refractoriness

- The repeated failure to raise the platelet count by transfusion of standard platelets
- One hour CCI < $5 \times 10^9$ m$^2$/L in two consecutive transfusions
Factors that affect the response to platelet transfusions

- The patient’s primary illness
  - Splenomegaly, leukemia, lymphoma, kidney failure

- The patient’s current clinical status
  - Sepsis, bleeding, fever

- The patient’s current treatment
  - Antibiotics, cytostatic drugs

- The platelet unit’s storage time

- Anti-platelet antibodies
  
  About one third of refractoriness cases have an immune component.

  Doughty et al., *Vox Sang*, 66:200, 1994
Immunity against allogeneic platelets

- Platelets carry ABO blood group antigens.
- There are 28 bi-allelic platelet-specific antigens (human platelet antigens, HPA)
  - Antibodies against HPA-1a or HPA-5b alleles are most common in cases of FNAIT.
- Platelets express HLA class I antigens HLA-A, -B and -C
  - Antibodies against HLA-A or -B alleles are the most common cause for immune-mediated platelet transfusion failure.
Antibody-mediated platelet destruction

Complement-mediated lysis

Phagocytosis by cells expressing Fc receptors

Lysis by Natural Killer cells (?)
How many HLA-typed donors are needed to provide HLA-matched platelets to all patients?

  - 5 completely matched donors for 80% of all patients
    - Japan: 5000, Europe: 18000, North America: 21000

  - Covering the transfusion needs for most patients (completely matched or one cross-reacting allele)
    - 1000-3000

- Bub et al., *Brazil J Hematol Hemother*, 38:1, 2016
  - 5 completely matched donors, or have one or two cross-reacting alleles
    - Complete match: 32000, 1x: 1710, 2x: 321

  - Number of donors to provide 70% of patients with platelets that are either completely matched or have one or two cross-reacting alleles
    - 12000
Acidic elution of HLA-molecules to create universal donor platelets

Sugawara S, Abo T, Kumagai K.:  
A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3.  

New approach to eliminate HLA class I antigens from platelet surface without cell damage: acid treatment at pH 3.0.  

Clinical advantages of acid-treated platelets

- They could survive and function in the circulation of patients with anti-HLA antibodies.
- They can be produced from random donor platelets and would be available in acute situations.
- They could be used as a complement to matched donor platelets if too few or no matching donors are available.
- They could be used in hospitals that lack the resources for organizing a sufficient pool of matched donors.
Transfusion of acid-treated platelets

Table 2. Clinical Results After Transfusion of HLA-Eluted Platelet Transfusions

<table>
<thead>
<tr>
<th>Author</th>
<th>Patient</th>
<th>HLA Elution Method</th>
<th>Number of Transfusions</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shanwell et al[1]</td>
<td>M, 40, multiple myeloma</td>
<td>Citric acid incubation, pH 2.8, 25 minutes 0°C, neutralization by plasma</td>
<td>2</td>
<td>Nose bleeding stopped, profuse gastrointestinal bleeding stopped, post-transfusion platelet increments obtained</td>
</tr>
<tr>
<td>Sivakumaran et al[5]</td>
<td>F, 79, myelodysplastic syndrome</td>
<td>Citric acid incubation, pH 2.8, 25 minutes 0°C, neutralization by plasma</td>
<td>1</td>
<td>No response</td>
</tr>
<tr>
<td>Novotny et al[1]</td>
<td>F, 39, acute myeloid leukemia</td>
<td>Citric acid incubation, pH 3.0, 10 minutes 0°C, neutralization by plasma</td>
<td>20</td>
<td>Gastrointestinal bleeding stopped, skin hemorrhages resolved, post-transfusion platelet increments obtained</td>
</tr>
<tr>
<td>F, 84, aplastic anemia</td>
<td></td>
<td>Citric acid incubation, pH 3.0, 10 minutes 0°C, neutralization by PAS II</td>
<td>1</td>
<td>No platelet increment, febrile transfusion reaction</td>
</tr>
<tr>
<td>F, 43, myelodysplastic syndrome</td>
<td>Citric acid incubation, pH 3.0, 10 minutes 0°C, neutralization by PAS II</td>
<td>1</td>
<td>Post-transfusion platelet increment from 12 to 18 × 10⁹/L.</td>
<td></td>
</tr>
<tr>
<td>M, 20, storage pool disease</td>
<td>Citric acid incubation, pH 3.0, 10 minutes 0°C, neutralization by PAS II</td>
<td>1</td>
<td>No post-transfusion platelet increment (from 60 to 57 × 10⁹/L); bleeding time 20 to 7 minutes</td>
<td></td>
</tr>
<tr>
<td>Castro et al[10]</td>
<td>F, 34, chronic myeloid leukemia</td>
<td>Citric acid incubation, pH 3.0, 10 minutes 0°C, neutralization by plasma</td>
<td>7</td>
<td>Bowel bleeding stopped, post-transfusion platelet increments obtained</td>
</tr>
</tbody>
</table>

Why do we try to revive this approach?

- The potential clinical advantages are big.
- No controlled clinical study has been performed so far.
  - In the case reports, different protocols for the acid-treatment were used.
  - Not all of them report, what quality controls they applied and whether they assessed the extent of HLA removal.
  - The status of the patients receiving the platelets differed strongly between the cases.
- There were no studies that investigated if the acid treatment prevented anti-HLA antibody-mediated platelet destruction.
HLA denaturation through treatment with low pH

- HLA-A, B, C
- β₂-microglobulin
- free HLA heavy chain

- isotype controls
- untreated platelets
- control-treated platelets
- acid-treated platelets
Platelet Immunofluorescence Test (PIFT)

Based on the method described by von dem Borne et al. 1978 Br J Haematol

donor platelets + patient serum with antibodies detection with anti-Fc antibodies
Acid treatment prevents binding of anti-HLA antibodies
An assay to measure antibody-mediated complement activation
Anti-HLA antibody-mediated complement activation

Calcein-labeled platelets were incubated for 10 min at 37 °C in PBS with the indicated components and then stained for C1q and C3c.
Acid treatment prevents anti-HLA antibody-mediated complement activation

Calcein-labeled platelets were incubated for 10 min at 37°C in PBS with the indicated components and then stained for C1q and C3c.

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3rd September 2018
Acid treatment prevents anti-HLA antibody-mediated complement activation

Calcein-labeled platelets were incubated for 10 min at 37°C in the presence of anti-HLA antiserum and active complement and then stained for C1q and C3c.
Phagocytosis assays with labelled platelets

Monocytes  Platelets + patient serum  Phagocytosis

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Phagocytosis assays with labelled platelets

Platelets readily attach to monocytes.

So, the problem is to distinguish between platelets on the surface and inside the monocytes.
Phagocytosis assays with labelled platelets

Trypsin/EDTA

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Phagocytosis assays with pHrodo

- pHrodo-labeled platelets + monocyte

- Adhesion, but no phagocytosis
  - Platelets on the surface: dim fluorescence

- Adhesion and phagocytosis
  - Platelets in lysosomes: bright fluorescence

- pH in lysosomes: about 4.8

```
0  50 100 150 200 250
# Cells
pHrodo
```

- Platelets in PBS
- Platelets at pH 2.9

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Anti-HLA antibody-mediated phagocytosis

Platelets were incubated with a mouse monoclonal anti-HLA-A,B,C antibody washed and then added to PBMC at a 10:1 ratio. Gated on CD14+ monocytes.
Acid treatment reduces anti-HLA antibody-mediated phagocytosis

Platelets were incubated with a mouse monoclonal anti-HLA-A,B,C antibody washed and then added to PBMC at a 10:1 ratio. Gated on CD14+ monocytes.
Acid treatment reduces anti-HLA antibody-mediated phagocytosis

pHrodo bright events are gated based on the respective cytochalasin D-treated samples. Plotted are means and SD of six combinations of four different platelet concentrates and two donors in four experiments.

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Summary I

- Acid treatment for 5 min reduces the level of HLA-A, B, and C on the platelet surface by 75 - 80%.

- Treated platelets were protected from anti-HLA antibody-mediated destruction in vitro:
  - Binding of anti-HLA IgG from all donors tested is prevented.
  - The complement system is not activated by anti-HLA antibodies.
  - Anti-HLA antibody-mediated phagocytosis is diminished.
A mouse model for platelet transfusion

1. Acid treatment
2. Fluorescent labelling

Human platelets

Blood sampling at different time-points

Untreated
Control-treated
Acid-treated
CFSE

I.v. injection

Immune-deficient mouse (NSG)
Survival of acid-treated platelets in the circulation
Acid-treated HLA-A2-positive platelets escape anti-HLA-A2-mediated destruction
Acid-treated HLA-A2-positive platelets escape anti-HLA-A2-mediated destruction

Before injection of antibody

2h after injection of antibody

% of injected platelets

0 2 4 6

time [h]

control-treated
acid-treated
untreated HLA-A2+
untreated HLA-A2-

anti-HLA-A2
Summary II: A mouse model for platelet refractoriness

- Acid-treated platelets have a lower recovery than untreated one hour after injection.
- The remaining platelets can still be detected after 20 h.
- Acid-treated platelets are protected from anti-HLA antibody-mediated clearance.
Clinical study

- Refractory patients with anti-HLA antibodies, for which no completely matched platelets are available
- Excluding patients with bleedings, fever or infections
- Primary question: Are acid stripped platelets superior to standard (non-matched) platelets?
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