Flow Cytometry in Transfusion Medicine

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December 28, 2007
Flow cytometry in the clinical laboratory

- Lymphocyte immunophenotyping in HIV infection
- Immunophenotyping of leukemias and lymphomas
- Enumeration of CD34+ stem cells
- DNA ploidy and content analysis
- Paroxysmal Nocturnal Hemoglobinuria
Non traditional applications

- Red blood cell antigen and antibody detection (pre-transfusion testing)
- Detection of feto-maternal hemorrhage
Pre-transfusion testing
# The ABO Blood System

<table>
<thead>
<tr>
<th>Blood Type (genotype)</th>
<th>Type A (AA, AO)</th>
<th>Type B (BB, BO)</th>
<th>Type AB (AB)</th>
<th>Type O (OO)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red Blood Cell Surface Proteins (phenotype)</strong></td>
<td><img src="image" alt="Type A" /></td>
<td><img src="image" alt="Type B" /></td>
<td><img src="image" alt="Type AB" /></td>
<td><img src="image" alt="Type O" /></td>
</tr>
<tr>
<td>A agglutinogens only</td>
<td>B agglutinogens only</td>
<td>A and B agglutinogens</td>
<td>No agglutinogens</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Plasma Antibodies (phenotype)</strong></th>
<th><img src="image" alt="Type A" /></th>
<th><img src="image" alt="Type B" /></th>
<th><img src="image" alt="Type AB" /></th>
<th><img src="image" alt="Type O" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>b agglutinin only</td>
<td>a agglutinin only</td>
<td>No agglutinin</td>
<td>a and b agglutinin</td>
<td></td>
</tr>
</tbody>
</table>
ABO Typing

- **Forward Type**
  - Add patient cells to known antisera (anti-A, Anti-B)
  - Determines antigens present on RBCs

- **Reverse Type**
  - Add patient serum to known cells (A cells, B cells)
  - Determines antibodies present in serum
Pre-transfusion Testing

- **Type and Screen**
  - Determine ABO & Rh (D) type
  - Screen for clinically significant alloantibodies
    - Rh blood group – Anti-C, anti-c, anti-E, anti-e
    - Duffy blood group – Anti-Fy(a), anti-Fy(b)
    - Kidd blood group – Anti-Jk(a), anti-Jk(b)
    - Kell blood group – Anti-K, anti-k
  - Mix 2-3 cell types (known antigens) with patient plasma
**Indirect Coombs test / Indirect antiglobulin test**

1. Recipient's serum is obtained, containing antibodies (Ig's).
2. Donor's blood sample is added to the tube with serum.
3. Recipient's Ig's that target the donor's red blood cells form antibody-antigen complexes.
4. Anti-human Ig's (Coombs antibodies) are added to the solution.
5. Agglutination of red blood cells occurs, because human Ig's are attached to red blood cells.

Positive test result
Gel Technology
Problems in Pre-transfusion Testing

- Tube Testing
  - Labor intensive
  - Not amenable to automation
  - Results operator dependent

- Gel Testing
  - Higher costs
  - Slower...not good for emergency testing
  - May not detect all weak antibody-antigen interactions
Pre-transfusion testing via Flow

- Compatibility testing
  - ABO blood group and D typing
  - Antibody screen
  - Identification of alloantibodies
First Hurdle in the application of Flow Cytometry to transfusion medicine

Red cell agglutination is a big problem...

**Solutions**

1. **Mechanical**
   - Vigorous pipetting and vortexing
   - Small bore needle

2. **Chemical treatment**
   - Glutaraldehyde, formaldehyde, dimethylsulphoimidate

3. Use secondary antibody that does not cause agglutination

4. Filter plate technique

5. Low speed centrifugation
ABO and D Typing

- Study by Roback et al.
- Compared ABO typing by flow cytometry with tube method and column agglutination technology (gel)
  - 222 random patient samples
### TABLE 1. FC immunohematology protocols for tube and filter plate tests

<table>
<thead>
<tr>
<th>Step</th>
<th>RBC A,B</th>
<th>RBC Rh(D)</th>
<th>Serum α-A,B</th>
<th>Allocantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Add RBCs</td>
<td>2% patient RBCs (25 µL)</td>
<td>2% patient RBCs (25 µL)</td>
<td>3% A, B, O RBCs (30 µL)</td>
<td>3% screening RBCs (25 µL)</td>
</tr>
<tr>
<td>2. Add primary Ab</td>
<td>Mse α-A or α-B (50 µL)</td>
<td>FITC Hum α-D (50 µL)</td>
<td>Patient plasma (50 µL)</td>
<td>Patient plasma (50 µL)</td>
</tr>
<tr>
<td>3. Add potentiator</td>
<td>NA</td>
<td>20% PEG (100 µL)</td>
<td>NA</td>
<td>20% PEG (100 µL)</td>
</tr>
<tr>
<td>4. Incubate</td>
<td>RT × 2 min</td>
<td>37°C × 5 min</td>
<td>NA</td>
<td>37°C × 5 min</td>
</tr>
<tr>
<td>5. Wash</td>
<td>Saline × 4 (200 µL)</td>
<td>Saline × 4 (200 µL)</td>
<td>Saline × 4 (200 µL)</td>
<td>Saline × 4 (200 µL)</td>
</tr>
<tr>
<td>6. Add secondary Ab</td>
<td>PE-α-Mse IgM (100 µL)</td>
<td>NA</td>
<td>PE-α-Hum IgM (100 µL)</td>
<td>PE-α-Hum IgG (100 µL)</td>
</tr>
<tr>
<td>7. Incubate</td>
<td>RT × 5 min</td>
<td>NA</td>
<td>RT × 5 min</td>
<td>RT × 5 min</td>
</tr>
<tr>
<td>8. Wash</td>
<td>Saline × 2 (200 µL)</td>
<td>NA</td>
<td>Saline × 2 (200 µL)</td>
<td>Saline × 2 (200 µL)</td>
</tr>
<tr>
<td>9. Disperse RBCs</td>
<td></td>
<td>Vigorous pipetting and vortexing (as necessary)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ABO and D Typing; Results

- **Accuracy**
  - FC - 99.1%
  - Tube - 95%
  - Gel - 91.9%

- **Conclusions**
  - FC performed as well as tube & gel for ABO & D typing
  - FC performed better for some samples
    - Rouleaux
    - Autoantibodies
    - Weak alloantibodies
    - Mixed-field reactions
Alloantibody Detection

239 random patient samples
  188 with alloantibodies
  51 with no alloantibodies

Accuracy:
FC - 99.5%
Tube - 94.7%
Gel - 98.9%

Subpopulation Detection

Further Study


- Personal Cell Analyzer (PCA-96)
  - A capillary cytometry system which can automatically acquire samples in a 96-well plate format
  - Low speed centrifugation used to separate RBCs
Results

- **ABO & D Typing Accuracy**
  - PCA-96 - 98.7% (229 samples)
  - Gel – 97.4%

- **Alloantibody Screens**
  - PCA-96 – 99.1% (213 samples)
  - Gel – 99.5%
  - FC was superior in detecting mixed-field reactions (p < 0.005)
Results, cont.

- FC showed good well-to-well and day-to-day reproducibility
- FC can detect alloantibodies with both homozygous & heterozygous target RBCs

Flow cytometry is an accurate and sensitive method of pre-transfusion testing and is a promising technique for fully automated testing in the blood bank.
Use of Flow Cytometry to Measure Feto-maternal Hemorrhage
Hemolytic Disease of the Newborn

- **Erythroblastosis fetalis**
- Alloimmune condition that develops in a fetus
  - IgG antibodies produced by the mother (antigen negative) cross the placenta and attack fetal red blood cells (antigen positive)
    - Usually due to anti-D antibodies
    - Can be caused by any red cell alloantibody (anti-C, anti-E, anti-K)
  - The red cells are hemolyzed and the fetus can develop reticulocytosis and anemia
- Fetal disease ranges from mild to very severe
  - Fetal death from heart failure (hydrops fetalis) can occur
Hemolytic Disease of the Newborn

- Alloimmunization
  - Mother forms antibody to antigen she is missing
    - Prior transfusion
    - Prior pregnancy*
      - Mother exposed to fetal blood during delivery
      - Mother exposed to fetal blood as a result of trauma (MVA)
A reduction in red blood cells leads to anemia, a condition marked by weakness and fatigue. Severe anemia can lead to heart failure and death. The breakdown of red blood cells also causes the formation of bilirubin, the build-up of which can lead to jaundice and possibly brain damage.

An Rh positive father and Rh negative mother may conceive an Rh positive baby.

In a subsequent pregnancy with an Rh positive baby there is the risk that it will develop Rh disease. Even though the blood circulation of the mother is separate from that of the child, the antibodies in her system can cross the placenta, enter the bloodstream of the baby, and cause its red blood cells to be killed.

This usually isn’t a problem if it’s the mother’s first pregnancy with an Rh positive child, because her blood circulation is separate from that of the baby.

The mother’s immune system recognizes the cells as foreign and develops antibodies against them.

At birth, or after an abortion or miscarriage, Rh positive blood cells from the baby enter the mother’s bloodstream.
How can we determine if a mother has been exposed to fetal blood?

Answer: Mother’s RBCs contain different hemoglobin than baby’s RBCs.
Hemoglobin Molecule

- α chain
- iron
- heme group
- β chain

red blood cell

helical shape of the polypeptide molecule
Hemoglobin Review

- **HbA** - >90% of total adult Hb
  - α2β2
- **HbA₂** - <3.5% of total adult Hb
  - α2δ2
- **HbF** – 1% of total adult Hb; 80-100% of total fetal/newborn Hb
  - α₂γ₂
Relative amounts of the several globin chains (ε, α, γ, β, and δ) present during fetal development and the first year of life.
Identification of Feto-maternal Hemorrhage

- Kleihauer-Betke Test
  - Cytochemical test
  - RBCs containing fetal hemoglobin (HbF) are resistant to acid elution
    - Peripheral smear stained with acid hematoxylin
    - Washed with citrate phosphate buffer
Kleihauer-Betke Test

- Used to quantify amount of Rh immune globulin (RhoGAM) needed to prevent Rh alloimmunization

- Problematic
  - Subjective
  - Imprecise
  - Operator dependent
  - Cannot distinguish maternal cells containing HbF and fetal cells
Identification of Feto-maternal hemorrhage by Flow Cytometry

- Quantification of fetal cells identified by specific blood group antigen
  - Anti-D antibodies
  - Antibodies to HbF
    - Some HbF is expressed in all adults
    - Slightly lower HbF expression in adult cells vs. fetal cells
- Fetal Cell Count Kit
Fetal Cell Count Kit

- Mouse monoclonal anti-HbF
- Polyclonal antibody to carbonic anhydrase
  - Enzyme only fully expressed in RBCs after birth
    - Adult levels at 6 months to 1 year of age
- Allows discrimination & quantification of 3 cell populations
  - Adult RBCs (HbF - , CA +)
  - Fetal RBCs (HbF +, CA -)
  - Adult F cells (HbF +, CA +)
Evaluation of Fetal Cell Count Kit


- Evaluated 455 pregnant or post-partum women & 124 artificial mixtures of adult and 0.01-5% fetal RBCs
  - Compared results of Kleihauer-Betke Test and Flow Cytometric Analysis (Fetal Cell Count Kit)
FL-1 = Carbonic Anhydrase-FITC
FL-2 = HbF-PE
Clinical Application

- RhoGAM injection (300µL) prevents alloimmunization due to 30 mL feto-maternal hemorrhage (0.03% fetal RBCs)
- Quantification of hemorrhage by Fetal Cell Count Kit has detection limit of 0.02% (200,000 cells gated) or 0.03% (100,000 cells gated)
Conclusions of Study

- Fetal Cell Count Kit is more precise than Kleihauer-Betke test for quantification of feto-maternal hemorrhage
  - Greater number of cells counted in shorter period
  - More objective
  - KBT can overestimate number of fetal cells
    - Increased amounts adult HbF cells
    - Erroneous low count of maternal cells
    - Very technician dependent!
  - KBT underestimates sizes of large feto-maternal bleeds
Other applications for flow in transfusion medicine

- Quantitation of residual WBCs after filtration of blood products
- Detection and quantification of RBC-bound IgG, IgM & complement (DAT)
  - Diagnosis of autoimmune hemolytic anemia
- Detection of antibody bound to platelets
- Determination of RBC survival after transfusion
- Phenotyping of recipient RBCs after transfusion
- Detection and quantification of minor RBC populations
  - McLeod carriers
  - Monitoring of bone marrow transplantation engraftment
- Quantification of RBC blood group antigen density