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**Immunohaematology**

**CLS 441**

**Department of clinical laboratory sciences**

**CLS 441 – Immunohaematology**

**OUTLINES OF PRACTICAL CLASSES**

1. Introduction to blood bank
2. Introduction to ABO grouping
3. Washing cells and making red cell suspensions
4. ABO grouping tube method (cell grouping)
5. Rhesus grouping
6. Rhesus grouping
7. Rhesus phenotyping
8. Other blood group typing
9. Techniques to detect IgG antibodies
10. Direct Coomb's Test
11. Indirect Coomb's Test
12. Techniques to detect IgG antibodies
13. Albumin technique
14. Enzymes technique
15. LISS
16. Revision + Mid-Term exam
17. Blood collection
18. Pre-transfusion compatibility testing
19. Compatibility testing
20. Non-urgent cases
21. Urgent + non-urgent cases
22. Antibodies screening + identification.
23. Antibody elution
24. Titration of antibodies
25. Other methods of serological testing –gel method-
26. Revision
27. Final exam

**Washing Cells and Making Red Cell Suspension**

Suspension of washed red cells is needed for all haemagglutination tests.

The cells must be washed at least once in order to remove plasma, weak cell suspensions are used in haemagglutination tests since the ratio of serum to cells affects the sensitivity of most tests – a minimum number of antibodies must bound to RBC's in order to bring about agglutination.

Materials:

1. Sample of blood
2. Saline
3. Test tubes 75x12 mm

Method:

1. Place 0.2 -0.5 ml of blood into the tube (2-3 drops).
2. Fill the tube with the saline.
3. Centrifuge at 200 G for 1-2 minutes until the RBC's are packed. (G=relative centrifugal force)
4. Decant the supernatant.
5. Tap the tube to resuspend the RBC's in the residual fluid. This constitutes one wash. Repeat step 2-5 at least twice. The last wash should always have a clear supernatant with no signs of haemolysis.
6. To make a 5% cell suspension add 1 volume of the packed RBC's to 19 volumes of saline.
7. To make a 3% suspension, add 1 volume packed RBC's to 32 volumes of saline.

**Grading**

The following system can be used as a guideline:

Table.1. Grading system

|  |  |
| --- | --- |
| C or ++++ | Complete agglutination; one clump of agglutinated cells. Score: 12 |
| V or +++ | Visual agglutination; several large clumps of cells. Score: 10 |
| ++ | A few large clumps seen with the naked eye. Score: 8 |
| + | Small evenly distributed clumps seen more easily under the microscope. Score: 5 |
| ± | Microscopic clumps of 5-10 cells and mainly unagglutinated cells. Score: 3 |
| W | Weak; 3-5 cells per clump. Score: 2 |
| 0, neg or –ve | Negative; no agglutination observed. It is pre- preferable not to use the minus sign '-' to record a negative result but 0 or neg. |
| L | Haemolysis; means a positive reaction. Total haemolysis should be scored as 12 and partial haemolysis as 10. |
| MF | Mixed field reaction; a mixture of agglutination and unagglutinated cells. |

**ABO Typing**

ABO typing is usually performed by saline techniques in tubes or micro-plates by testing the patients or donor's red cells with anti-A, anti-B and the serum or plasma with A cells, B cells and O cells.

Tube method:

This is the method of choice and is particularly suitable when grouping large numbers of samples. Both patient's cells and serum must be grouped and the two results compared and control must be done.

1. Cell grouping:
2. Place a drop of each grouping serum in three tubes labeled anti-A, anti-B and anti-AB in a rack.
3. Add one drop of 5% patient cells to all three tubes.
4. As controls set up 3 rows of 3 tubes each (9 tubes). Add one drop of anti-A, anti-B and anti-AB into 1st, 2nd and 3rd horizontal rows respectively. Then pipette 1 drop of A cells, B cells and O cells (3-5% suspensions) in the 1st, 2nd and 3rd vertical rows respectively.
5. Mix all tubes and read agglutination after one and half to two hours at room temperature.
6. Serum grouping:
7. Pipette 1 drop of patient serum in 4 tubes labeled A cells, B cells, O cells and patient cells.
8. Add 1 drop of 5% suspension of A, B, O cells and patient cells to the above tubes accordingly.
9. Mix and read agglutination after one and half to two hours at room temperature.

NOTE:

There should be no discrepancy between results of cell and serum grouping expect in the following:

1. Infants below 3 months old in whose sera antibodies are absent or not fully developed.
2. Cases of group A2 or A2B with anti-A1 antibodies.

Table.2.The ABO antigens and antibodies

|  |  |  |  |
| --- | --- | --- | --- |
| Blood group | Antigens | Antibodies normally present in serum | Antibodies occasionally present in serum |
| A1B | A + A1 + B | None | Anti-H |
| A2B | A + B | None | Anti-A1 (25-30% of sera) |
| A1 | A + A1 | Anti-B | Anti-H |
| A2 | A | Anti-B | Anti-A1 (1-2% of sera) |
| B | B | Anti-A + Anti-A1 | Anti-H |
| O | None | Anti-A + Anti-A1 + Anti-B | None |

**Rh (D) Typing**

In the Rh blood group system, cell typing involves only detection of antigen D using potent using potent anti-D reagent.

Rh (d) typing may be performed on a slide (tile) or in tubes using saline-reactive anti-D or chemically modified anti-D (IgG type low protein).

**Methods:**

1. Rh(D) typing (slide technique):
2. Place 1 drop of anti-D on labeled slide
3. As control place 1 drop of albumin or any control medium on another labeled slide.
4. To each slide added 2 drops of the tested cells (40-50%) cells suspended in plasma serum.
5. Mix and read within 2 minutes.

Interpretation:

A positive test has agglutination with anti-D. A negative test has smooth suspension as the cells.

Causes of False reaction in slide tests:

1. False positive results may be caused by the following:
2. Drying on the slide
3. Small fibrin clots
4. False negative results:
5. Too weak saline-suspended cells
6. Weakly active cells (old cells)
7. Using wrong reagent.
8. Rh typing (tube technique):
9. Place one drop of anti-D in a tube labeled (test) and in each control tube positive and negative (albumin can be used as a control).
10. Add one drop of 5% test cells suspension in tube labeled (test).
11. Add one drop of O Rh positive cells into the positive tube and O Rh negative cells into negative tube.
12. Mix all tubes and incubate at 37ºC for 15 minutes.
13. Check for agglutination.

A positive test means Rh positive. In case of negative results, spin the negative tube for 15 seconds at 1000 G and read again. A positive result means Rh positive cells. In case of negative results, do Coomb's test to check for Du antigen or to confirm negative Rh cells.

**Rhesus phenotyping**

1. Using (saline antisera):
2. Prepare 2% cell suspension.
3. Place 1 drop of anti-JD, anti-C, anti-c, anti-E, anti-e in five tubes.
4. Add 1 drop of 2% cell suspension of patient cell to all tubes.
5. Mix and incubate at 37ºC for one and half to two hours.
6. Read agglutination macro and microscopically.
7. Determine the most probable phenotype.
8. Using (antisera with incomplete antibodies):
9. Set up exactly the above procedure from step1-4.
10. Wash all tubes and do Coomb's test.
11. Read agglutination after Coomb's test.
12. Determine the most probable phenotype.

**Antiglobulin Test**

**Direct Coomb's test:**

1. 1 drop of 5 % cell suspension
2. 2 drops of AHGS
3. Centrifuge for 15 seconds
4. Read the agglutination

**Indirect Coomb's test:**

1. 2 drops of serum from EDTA sample
2. 1 drop of O+ cells
3. Incubate 20-30 minutes at 37ºC
4. Wash 4 times
5. 1 drop of AHGS and spin for 15 minutes
6. Read the agglutination

**Antiglobulin Techniques**

This technique is used for antibody screening, antibody detection, cell typing with certain antisera and for compatibility testing. The indirect Antiglobulin test (I.A.T) is performed in four stages:

1. The incubation of test RBCs and test serum at 37ºC (sensitization phase)
2. Washing of RBCs to remove excess protein and free IgG in the suspension medium
3. The addition of anti-human globulin serum (AHGS)
4. The checking of negative results with IgG-coated control RBCs

Materials:

* 5% cell suspension in saline
* Serum or grouping reagent
* Test tubes
* Anti-human globulin serum (polyspecific or broad spectrum AHGS)
* Control cells: IgG-coated cells washed 4 times

Methods:

1. In a tube, mix 3-4 volumes of serum with 1 volume of cells
2. Incubate at 37ºC for 45-60 minutes
3. Examine for haemolysis and agglutination. If agglutination is observed, record as positive and do not proceed to 4. A positive result in such circumstances should not be interpreted as being due to IgG or C3 coating. Haemolysis should also be recorded; if partial proceed to 4.
4. Wash at least three times, automatically in a cell washer or manually. If washing manually, ensure that as much supernatant as possible is removed after each wash and that the cell button is totally resuspended in saline before proceed into the next wash, this is achieved by tapping or shaking from the cell button and then adding saline, with force, from a plastic wash bottle.
5. Add 2 drops of AHGS and mix.
6. Centrifuge the tubes at 1000G for 15-20 seconds.
7. Remove the tubes and read visually over a light source.
8. Record results.
9. If the test is negative, leave the tube at room temperature for 5 minutes, re-centrifuge and examine.
10. If the test is still negative, add 1 drop of IgG-coated RBCs.
11. Repeat steps 6 and 7.

A positive reaction indicates that the negative result in step 7 is valid, but if the control IgG-coated cells fail to agglutinate, then the test must be repeated.

**Albumin Technique**

This test is accomplished in two steps:

1. The cells and serum are incubated, so that the antibody binds to the appropriate antigen (sensitization)
2. Then, bovine albumin is added and this draws the RBCs closer together, so that the IgG antibodies sensitizing the RBCs can bind to adjacent cells forming agglutinates. RBCs uncoated with antibody remain unclumped with the concentration of bovine albumin used.

Materials:

* 3% red cell suspension
* Serum
* 20% bovine albumin (22% or 30% albumin can be used)
* Test tubes

Method:

1. Place two volumes of serum and one volume of 3% cells into the tube
2. Incubate for 30 minutes at 37ºC.
3. Centrifuge for 1 minute (200 G) at room temperature. Alternatively, incubate for 60 minutes and then centrifugation is not required.
4. Add one drop of albumin so that it slides down the inside of the tube. Do not mix.
5. Incubate for a further 15 minutes at 37ºC.
6. Read and record agglutination.

**Enzyme Technique**

This technique is used for antibody screening, antibody detection and cell typing with certain antisera.

Some antibodies, especially those with Rh, Kidd and Lewis specificities, will agglutinate or even lyse RBCs that have been treated with proteolytic enzyme such as papain, ficin, trypsin or bromelin. These enzymes remove some of the negatively charged molecules (mainly sialoglycoproteins) from the cell surface, allowing the RBCs to come close enoughfor IgG molecules to agglutinate them when suspended in saline. On the other hand, a different theory suggests that proteolytic enzymes enhance agglutination by removing part of the hydration layer surrounding the red cells.

Materials:

* Packed RBCs
* Serum
* Stock Lows papain diluted 1 in 10 in saline
* Test tubes

Method:

1. Papain premodification
2. In a test tube add 4 drops of 0.1% papain to 1 drop of packed cells
3. Incubate at 37º C for 12 minutes in a water bath. The time may vary with the batch of papain and needs to be standardized for each new batch.
4. Wash the treated cells twice in saline
5. Make up a 3% cell suspension in saline. These cells can be stored at 4ºC for up to 48 hours
6. Use of premodified cells
7. In a test tube mix 1 drop of the papain-treated 3% cell suspension and 1 drop of serum
8. Incubate at 37ºC for 20 minutes
9. Centrifuge at 200 G for 1 minute at room temperature
10. Alternatively, incubate for 60 minutes and then centrifugation is not required.
11. Examine

**Low Ionic Strength Indirect Antiglobulin Technique (LISS/IAT)**

In LISS techniques, lowering the ionic strength of the reaction medium increases the rate of uptake of most antibodies by antigen; hence incubation time can be reduced. However, if the volume of serum is increased from the recommended n a given LISS technique, the ionic strength of the mixture will increase and the benefits for the technique will be lost. It is therefore important not to deviate from the recommended procedure. Some Kell allo-antibodies react more easily under low-ionic strength conditions while antibodies of most specificities are enhanced. The first stage of agglutination, i.e. sensitizations, can be modified to make use of this enhancement in two different ways: by suspending the test RBCs in a low-ionic salt solution (LISS) or by using a low-ionic salt addition solution.

Materials:

* Test tubes
* LISS
* Test RBCs
* Serum
* AHGS
* Control Rh-positive cells pre-coated with anti-D and washed four times

Method:

1. Wash test RBCs twice in saline and then once in LISS
2. Resuspended test RBCs in LISS to a 3% suspension
3. In a tube, mix an equal volume of test serum and LISS-suspended cells (i.e. 2 or 3 drops from a Pasteur pipette or 100µL measured volume)
4. Incubate tubes at 37ºC for 15 minutes
5. Remove Tubes and examine for haemolysis and/or agglutination
6. Wash RBCs at least three times manually or in a cell washer
7. Add AHGS and spin for 15 seconds
8. Examine

**Pretransfusion Compatibility Test**

1. ABO grouping
2. Rh grouping
3. Antibody screening
4. Antibody identification
5. Cross-match

**Compatibility tests**

1. Place 1 drop of patient serum in each of three tubes labeled saline R.T (room temperature), saline 37 and albumin; and two drops in a fourth tube labeled Coomb's.
2. Add 1 drop of 5% donor cells to each of the first three tubes and 2 drops of 5% donor cells to the fourth tube.
3. Mix all tubes. Incubate the second + third+ fourth at 37ºC. Incubate the first tube at room temperature.
4. After an hour to one hour and half, add one volume of 22% albumin to the third tube and incubate for another 30 minutes.
5. If the results are negative at end of incubation period for the first three tubes, carry out Coomb's test on fourth tube.
6. If there is NO agglutination after Coomb's test, then blood is regarded as compatible.

**Antibody Screening**

1. Antibody detection:
2. Place 2 drops of patient serum in a tube
3. Add 1 drop of pooled O cells 2%
4. Mix and incubated at 37ºC for 1-1½hour
5. Read agglutination microscopically and confirm negative results by Coomb's test.
6. Identification of antibodies:
7. Place a drop of patient sera in a row of tubes
8. Add 1 drop of cells using the panel of cells supplied
9. Mix and incubate at 17ºC for 1-1½ hour
10. Confirm negative results by Coomb's technique
11. Identify antibodies using table provided

**Titration**

The titre of an antibody is a semi-quantitative reflection of its concentration in a given serum. Titres can also help to determine the relative strength of a given antigen in a different cell samples (e.g. MM cells usually give a higher titre with a given anti-M than MN cells). One of the most useful applications of titrations is in the follow-up of samples from antenatal patients with antibodies that may cause haemolytic disease of the newborn.

Method:

Two-fold master dilutions are prepared in 75x12 mm tubes as follows:

1. Into 9 tubes, labelled 2 to 10, deliver a constant volume (e.g. 0.5 ml) of isotonic saline or AB serum.
2. Tube No.1 will contain a fixed volume of neat test serum (e.g. 0.5 ml). Into tube No.2, add the same volume of test serum as in 1(i.e. the same as the volume of saline or AB serum). This is mixed several times, using a clean pipette tip, to give a 1 in 2 dilution.
3. Remove a fixed volume from tube No.2 and add to tube No.3, mix to give a 1 in 4 dilution
4. The process is repeated, with a clean pipette tip for each dilution, to the end of the row of tubes.
5. An aliquot of each dilution is then used and tested against the appropriate cells, depending upon which technique has been selected for the titration (e.g. saline, IAT). The same pipette tip may be used if the highest dilution, i.e. from tube No.10, is dispensed first.

Techniques are performed, read and scored as described previously (e.g. saline, IAT). The titre is the reciprocal of the highest dilution that shows a positive reaction,(i.. if the 1 in 64 dilution gives appositive reaction but the 1 in 128 dilution dose not, the titre is 64). However, a titration score provides a better idea of the strength of an antibody than a titre. For example, when comparing two sera containing antibodies of the same specificity and using cooling system on page.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Test serum | Dilutions | | | | | | | | | Score |
|  | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 156 |  |
| X | C | C | +++ | ++ | + | + | (+) | - | - | 55 |
| Y | C | C | C | +++ | +++ | ++ | + | - | - | 71 |

Although both tests sera have a titre of 64, serum Y contains the stronger antibody with a score of 71, compared with serum X with a score of 55. As stated previously, a difference in score greater than 10 is significant.

**Antibody Elution**

Elution technique is used to remove incomplete antibodies which have coated RBCs in vivo. It is used for patients with autoimmune haemolytic anaemia, haemolytic transfusion reactions or in haemolytic diseases of the newborn.

Antigen-antibody binding is a reversible reaction which depends on weak physic-chemical forces holding the molecules together. To remove or elute antibodies from antigens these forces can be broken by altering the ionic strength or PH of the system, or by using organic solvents or extreme temperature changes.

**Gel Method**

The original technique is based on the principle of gel filtration for separation of red blood cells from human blood. The column consists of special microtubes containing a dextran gel matrix. Red blood cells and serum or red blood cells alone are dispensed into the microtubes, incubated if necessary, and then centrifuged under strictly controlled parameters. The gel within the microtubes acts as a sieve, unagglutinated red blood cells form a pellet at the bottom of the microtubes, and agglutinated red blood cells are trapped in the gel. Reactions are easily visible and may be graded.

The aim of this technology is to standardize red blood cell agglutination reactions, and by trapping the agglutinates, to permit simple and reliable reading.



Advantages:

1. Stability of reactions
2. Easy to use
3. Results can be photocopied
4. Small sample volumes used
5. Decreased false positive reaction

Disadvantages:

1. Cost
2. Loss of "traditional" skills