MAIPA standard protocol for platelet antibody detection

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1 Principle

The monoclonal antibody immobilization of platelets (MAIPA) assay is a glycoprotein–specific enzyme immunoassay and therefore allows detection of specific platelet antibodies in human sera even if they contain mixtures of antibodies against different antigenic glycoproteins (including HLA class I) on platelets. Monoclonal antibodies which bind to epitopes of these immunogenic glycoproteins without inhibiting binding of the human antibodies to be studied are required. Intact test platelets are incubated with the human serum under investigation, washed and incubated with the glycoprotein-specific monoclonal antibody. Platelets are again washed and solubilized in buffer containing Triton X-100 as detergent. The glycoprotein "labelled" with monoclonal antibody is then immobilized to the plastic surface of a microtiter plate through goat anti-mouse IgG. A human antibody attached to the same glycoprotein can be detected with enzyme-labelled goat anti-human IgG. The assay may also be used for the determination of glycoprotein-specific PAIgG on patients' autologous platelets.

The technique described here is modified in two aspects [1] compared to the originally published procedure [2]:

- Human sera sometimes contain antibodies reacting with mouse immunoglobulins. These "anti-mouse" Igs may bind directly to the monoclonal antibody and thus yield false positive results. In order to avoid this, test platelets are better incubated first with the human serum, washed once and then incubated with the monoclonal antibody[1]. This sequence of incubation with human and mouse (monoclonal) antibody must not be reversed.

- Sensitivity and signal-to-noise ratio are considerably enhanced by use of peroxidase (HRP)-labelled anti-human IgG[1] instead of alkaline phosphatase–labelled conjugates. This allows reduction of the absolute number of platelets per assay to 20×10^6. Moreover, platelets are now more easily washed with 100 μl instead of 500 μl isotonic saline.

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2 Description of the procedure

2.1 Indirect MAIPA

1. 20×10^6 platelets[1] suspended in approximately 50 μl of isotonic saline are centrifuged (microtiter plate centrifuge, 1 200 x g, 2 minutes) in wells of a round-bottomed microtiter plate, for removal of the supernatants the microtiter plate is flicked off into a sink, blotted to a paper towel and platelets are then resuspended in 30 μl of BSA 2%. For resuspension of the platelets in this step and in later washing steps, the microtiter plate is rotated horizontally (900 rotations per minute, rotating radius 3–5 mm) for 2 minutes. Then, 5-50 μl of the human serum to be investigated is added.

2. Following an incubation at 37 °C for 30 minutes, platelets are washed one time with 50 μl isotonic saline, resuspended again in 30 μl PBS BSA 2%, and 10 μl of the glycoprotein-specific monoclonal antibody (IgG concentration approximately 10-20 μg/ml) are added.

3. The platelets are again incubated (30 minutes, 37 °C) and washed three times. Therefore platelets are resuspended thoroughly in 50 μl of isotonic saline as described in step 1.

4. After the last centrifugation of the microtiter plate, the pellet is resuspended in 100 μl of solubilization buffer. Platelets/solubilization buffer are transfered to a 1.5 ml Eppendorf reaction tube. Platelets are allowed to lyse at 4 °C for 30 minutes, the lysates are then centrifuged ($15,000\times$ g for 30 minutes at 4 °C) to remove particular material.

5. Following centrifugation, the upper 50 μl of the supernatants are taken off and are diluted in 200 μl of TBS wash buffer; 100 μl of these dilutions are pipetted into wells of the microtiter plate coated with goat anti-mouse IgG.

6. After incubation (90 minutes, 4 °C), the wells are tipped out and are washed four times with 200 μl TBS wash buffer, incubated (120 minutes at 4 °C) with 100 μl of HRP labelled goat anti-human IgG diluted in TBS wash buffer.

7. Finally, the wells are washed five times with 200 ml wash buffer and incubated (15 minutes in the dark at room temperature) with 100 ml of substrate solution. The enzyme reaction is stopped by adding 50 μl of 2.5 m $\mathrm{H_2SO_4}$. The reaction is measured in a photometer at 492 nm.

   Results are expressed as ΔOD values:

$$\Delta OD = OD_{test} - OD_{blank}$$

   (OD_{blank}: first incubation on the microtiter plate with TBS wash buffer instaed of lysate)

2.2 Modifications/comments

The concentration of the enzyme-labelled conjugate is to be determined for each lot of conjugate. To assess the optimal conjugate concentration, the MAIPA assay is run with different normal human sera from normal donors with monoclonal antibodies against all glycoproteins studied. We select the highest conjugate concentration (i.e. the lowest conjugate dilution) which yields ΔOD values below 0.1 obtained with normal human sera.

Normally, test platelets are isolated from EDTA anticoagulated blood by differential centrifugation and may be stored at 4 °C in isotonic saline containing 0.1% $\mathrm{NaN_3}$. However, if test platelets are to be used for detection of

[1] isolated from EDTA anticoagulated blood by differential centrifugation

antibodies against the GP Ib/IX complex, the isolation procedure has to be modified: the GP Ib_α chain is easily cleaved by the platelet-borne Ca^{++}-dependent protease. Platelets are washed three times with isotonic saline containing 0.5% EDTA and are finally resuspended (500.000 platelets/μl) in isotonic saline with 0.1% NaN_3 and 50 μg/ml leupeptin added. These platelets may be used for 4-7 days when stored at 4 °C. The integrity of GP Ib_α should be checked frequently. In addition, leupeptin should be added to the solubilization buffer at a concentration of 100 μg/ml.

For detection of **HPA-15-specific antibodies**, fresh test platelets isolated the day before testing are isolated using PBS-BSA-EDTA for washing during the differential centrifugation steps. For each test a total of 100×10^6 platelets per assay are used in the test (required due to the low density of antibody binding sites).

2.3 Direct MAIPA

Step 1 in the "Indirect MAIPA" chapter is omitted. The procedure begins similarly as described in step 2: Patients' platelets are isolated from EDTA anticoagulated blood, 20×10^6 platelets are centrifuged in 1.5 ml (Eppendorf) reaction tubes, supernantants are sucked out and platelets are resuspended in 30 μl of PBS–BSA 2% and 10 μl of the glycoprotein–specific monoclonal antibody (cf. item 2) are added.

3 Reagents

Coating buffer 1.59 g Na_2CO_3; 2.93 g $NaHCO_3$; 0.2 g NaN_3 are dissolved in distilled water ad 1.000 ml, pH is checked and is adjusted to 9.6, if necessary.

Microtiter plate coated with anti-mouse IgG Goat anti-mouse IgG is diluted 1:500 in coating buffer, 100 μl of this dilution are pipetted into wells of a flat bottomed microtiter plate with high protein binding capacity and incubated at 4 °C overnight. 30 minutes before use, the wells are washed four times with TBS wash buffer, free binding sites on the plastic surface are blocked approximately 20 minutes at 4 °C with TBS wash buffer.

PBS–BSA 2% Dulbecco's phosphate buffered saline × 10 is diluted 1:10 in distilled water, pH is adjusted to 7.2, bovine serum albumin is added to a concentration of 2%.

TBS wash buffer 1.21 g Tris is dissolved in 950 ml of isotonic saline, pH is adjusted to 7.4, 5 ml Triton X-100, 0.5 ml of Tween20, and 0.5 ml of 1 m $CaCl_2$ are added, isotonic saline ad 1.000 ml.

1 m $CaCl_2$ 21,9 g $CaCl_2 \cdot 6H_2O$ are dissolved in 100 ml of distilled water.

Solubilization buffer 1.21 g Tris are dissolved in isotonic saline, pH is adjusted to 7.4, 5 ml of Triton X-100 are added, isotonic saline ad 1.000 ml.

Substrate solution Four tablets of 1,2-phenylenediamine (OPD) (2 mg tablets, Dakopatts, Hamburg) are dissolved in 12 ml of distilled water in the dark. Immediately before use 5 μl of 30% H_2O_2 are added.

PBS-BSA-EDTA To 70 ml of Dulbecco's phosphate buffered saline x 10, 630 ml of distilled water are added, 5 ml of bovine serum albumin 30% and 10 ml of 5% of Na_2EDTA (Titriplex III); pH should be 7.2.

Immunochemical reagents HRP labelled goat anti-human IgG: Jackson no: 109-035-098; goat anti-mouse IgG: Jackson no: 115-005-071.

References

[1] Kiefel V. The MAIPA assay and its applications in immunohematology. Transfusion Medicine 1992;2:181–188.

[2] Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet reactive antibodies. Blood 1987; 70:1722–1726.