CHAPTER II

MATERIALS AND METHODS

Materials

1. Reagents

Broad spectrum antiglobulin serum	(Ortho Diagnostics)
Anti-IgG serum	(Ortho Diagnostics)
Anti-complement serum	(Ortho Diagnostics)
Bovine albumin (30%)	(Ortho Diagnostics)
Reagent Red Cells	(Blood Bank Laboratory., Ramathibodi Hospital)

2. Chemicals

Penicillin G sodium	(Merck Sharp & Dohme)
Aldomet (alphamethyldopa)	(Merck Sharp & Dohme)
Disodium hydrogen phosphate	(May & Baker)
Potassium dihydrogen phosphate	(May & Baker)
Sodium chloride	(E. Merck)
Trisodium citrate	(May & Baker)
Citric acid	(British Drug House)
Tris (hydroxymethyl)	[illegible])
methylamine	(British Drug House)
Papain	(Difco)
2-Mercaptoethanol	(Sigma)

3. Glasswares

Test tube (10 x 75 mm)	(Pyrex)
(16 x 150 mm)	
Erlenmeyer flask 125 ml	(Pyrex)
Disposable pipette	

4. Instruments

Refrigerator (4°C)	
Deep freezer (-20°C)	
Incubator (37°C)	(Clay Adams)
Centrifuge (Serofuse)	(Clay Adams)
Centrifuge	(IEC, Model HN)
Micropipette 100 microliters	(Oxford Laboratories)

Methods

1. General method

Blood are obtained by venepuncture, either soon after clotted, or after overnight storage of the blood at 4°C, a direct antiglobulin (Coombs) test is performed. Sera obtained from clotted blood are tested on the day of collection or kept at -20°C until tested.

2. Detection of antipenicillin and anti-methyldopa antibody

2.1 Antipenicillin antibody detection

2.1.1 Sera specimens

Sera are obtained from 2,500 unselected patients, and 70 sera from patients with cardiovascular disease, many of whom are candidates for corrective cardiac surgery and received large

doses of penicillin. These sera are obtained originally for routine blood bank testing at the Division of Blood Banking and Immunohematology, Ramathibodi Hospital.

The sera of a group of healthy individuals donating blood are also detected for antipenicillin antibody for comparison with patient groups.

2.1.2 Preparation of penicillin treated (coated) red cells

1. Modified Alsever's solution

Dextrose	20.5 g
Trisodium citrate	8.0 g
Citric acid	0.5 g
Sodium chloride	4.2 g
Distilled water to make	1000.0 ml

2. Phosphate buffered saline (PBS) pH 7.2, 0.1 M

Sodium chloride	8.5 g
Disodium hydrogen phosphate	8.62 g
Potassium dihydrogen phosphate	2.48 g
Distilled water to make	1000.0 ml

3. Tris buffer pH 9.6

Tris	2.2114 g
EDTA disodium salt	0.2922 g
Sodium chloride	5.844 g
Distilled water to make	1000.00 ml

4. Penicillin solution

Dissolve 1 x 10^6 units (approximately 600 mg) of sodium penicillin G in 15 ml of Tris buffer pH 9.6

Method of preparation

1. Five ml of blood group O, Rh positive donor is drawn, mixed with an equal volume of modified Alsever's solution in a 15 x 160 mm glass test tube.

2. Centrifuge at 3400 rpm in a Sero-fuge (Clay Adams) for 1 min., removed plasma and red cells are washed three times in PBS.

3. To 1 ml of washed packed red cells, 15 ml of penicillin solution are added.

4. The mixture are incubated at room temperature (18°-25°C) for 2 hours with gentle mixing every 15 min.

5. An aliquot of the same red cells incubate under the same conditions but without penicillin serve as a control.

6. After the incubation period, treated red cells are washed three times with PBS, if some lysis of red cells occur during the incubation period, the cells are then washed until the supernatant is free of hemoglobin.

7. The treated red cells are suspended in a PBS at a concentration of 5% cells suspension and used for antipenicillin antibody detection. The penicillin treated red cells are stored in PBS at 4°C for no longer than one week.

2.1.3 Techniques of antipenicillin antibody detection

1. One drop (0.03 ml) of 5% penicillin treated red cells is added to 0.1 ml of sera or eluates in a 10 x 75 mm glass test tube.

2. Mix and centrifuge at 3400 rpm for 20 sec., read macroscopically; agglutination is graded from 0 to 4+

3. Move tubes to incubate at 37°C incubator for 30 min., and then centrifuge for 20 sec.

4. After the agglutination is graded, the cells are washed three times with PBS.

5. One drop of a broad spectrum antiglobulin (Coombs) seru is added to the cell bottom, after centrifuging for 20 sec., the agglutination is read macroscopically.

All sera and eluates are tested in parallel against untreated red cells. In positive reaction, agglutination is observed only with penicillin-treated red cells.

2.2 Antimethyldopa antibody detection

2.2.1 Sera spectimens

Frozen sera or fresh sera from 32 hypertensive patients taking methyldopa (alpha-methyldopa, Aldomet) are tested for the presence of antibody to methyldopa.

2.2.2 Preparation of methyldopa solution

Weigh and pulverize 5 tablets of methyldopa. Dissolve and accurately weigh quantity of the powder equivalent to about 1.0 g of anhydrous methyldopa as completely as possible in

sufficient PBS to produce 100 ml. and filter. The final concentration of methyldopa solution is 10 mg/ml.

2.2.3 Techniques of antimethyldopa antibody detection

1. Prepare a 5% washed red blood cells suspension in PBS from normal blood group O, Rh positive.

2. One-tenth ml of washed red cells suspension, methyldopa solution are added to 0.1 ml of patient's serum or eluate in the PBS.

3. The tube contents are mixed, and incubated at 37°C for 30 min.

4. Following incubation, they are centrifuged at 3400 rpm for 20 sec. The supernatant is examined for hemolysis and the bottom for agglutination.

5. The cell suspension is washed three times in PBS and an antiglobulin test is performed.

Control tube contain washed red cells, methyldopa solution and serum from normal donor.

3. Determination of the immunoglobulin class of circulating antipenicillin antibody

3.1 Sera specimen

Ninety-nine patient's sera containing antipenicillin antibody are proceeded for the study of immunoglobulin classes of antibody by inhibition with 2 ME.

2.2 Preparation of 0.2 M solution of 2 ME.

Seven milliliters of 2-Mercaptoethanol is added

in phosphate buffered saline and make up to 500 ml.

3.3 Methods

3.3.1 A 0.2 ml of a 0.2 M solution of 2-ME in PBS is incubated with 0.2 ml of a two-fold dilution of serum in PBS for 1 hour

3.3.2 Serum similarly diluted with PBS are incubated simultaneously as a control.

3.3.3 Penicillin antibody titers are then determined by direct agglutination and by the indirect antiglobulin test using broad spectrum antiglobulin serum.

3.3.4 If antibodies are completely inhibited by 2-ME they are considered to be IgM antibodies; sera with residual IgG antibodies are titered by the indirect antiglobulin test.

3.3.5 For a control, 2-ME is used in the same manner with an incomplete anti-D antibody (IgG) and a high titer cold agglutinin anti-M (IgM).

Principle :

Inhibition of IgM with sulhydryl compounds (e.g. 0.02 - 0.2 M. 2-Mercaptoethanol at pH 7) splits it into 6-7 S subunits. IgM antibodies in serum treated in this way lose their ability to agglutinate red cells, although the subunits retain their ability to combine with antigen. The serological behaviour of IgG antibodies treated as described above is unaffected.

4. Determination of the incidence of a positive direct antiglobulin test

4.1 Blood specimens

Blood specimens obtain from two groups of patients, are divided according to the mechanism by which the drugs produced the positive direct antiglobulin test. These two groups are :

4.1.1 Group I, 99 patients who have antipenicillin antibody in their sera.

4.1.2 Group II, 32 hypertensive patients taking methyldopa and 24 patients with hypertension who have not received methyldopa served as the control group. These hypertensive patients are attending at Endocrine Clinic, Ramathibodi Hospital.

4.2 Methods

4.2.1 Prepare a 4% cell suspension in PBS of patient's red cells to be tested in a 10 x 75 mm glass test tube. Wash red cells at least three times with PBS. Completely removed supernatant PBS after the last wash and then one drop of broad spectrum antiglobulin serum is added. The antibody cell suspension is centrifuged for 20 sec. at 3400 rpm and the cell bottom is examined for agglutination.

4.2.2 Agglutination, if present, is graded from one plus to four plus reflecting the degree of agglutination as follows : One large clump, 4+; several large clumps with one or two

small clumps, 3+; many small clumps with little or no free cells, 2+; many small clumps with many free cells, 1+; a few small clumps coming off a rugged bottom, trace (wk), linear streaks of cells coming off a smooth bottom, negative (-)

4.2.3 The strength of antibodies on the red blood cells is expressed as the sum of scores. The reaction are graded from weakly positive to 4 plus are equal to a continuous gradient from 2 to 12 scores.

Quantitative Notation	Score
4+	12
3+	10
2+	8
1+	5
wk	2
0	0

4.2.4 If the test is positive, further direct antihumanglobulin has been performed by using monospecific antiserum, anti-IgG, and anti-complement serum.

4.2.5 The sera of all patients with positive direct antiglobulin test will be screened for the presence of irregular antibodies to blood groups system. Using pooled panel O cells as the reagent red cells, the screening tests are done in saline, albumin and enzyme (papain) at room temperature and at 37°C for 30 min. Broad spectrum antiglobulin serum is added after third washing of tested red cells.

Principle

The adverse drug reactions affecting the human red blood cells proceeded by immune mechanisms. Such immune mechanisms are recognized and studied by the powerful serologic method, the antiglobulin (Coombs) test.

There are two major types of red blood cell coating proteins. The first type, gamma globulins of 7S type, the immunoglobulin G (IgG) globulins that are detected by an immune agglutinating rabbit serum reagent prepared against these proteins. Antiglobulin (Coombs) tests of this type are referred to an anti-gamma (Anti-IgG) reactions. The second type of reaction detected the presence of certain components of the complement (C') system on the surface of the red cells. This pattern of reaction has been called "anti-nongamma" in the part but now more appropriately referred to as the anti-complement reaction. In addition, the term broad spectrum antiglobulin serum is an immune rabbit serum reagent. This reagent had multiple antibody specificities which detect the presence of human immunoglobulin and or complement components on red blood cells. If red blood cells acquire their antibody coating in vivo (that is are circulating in the coated state), the antiglobulin test is referred to as a positive direct test of either specificity. If, on the other hand, the technique is used to detect in vitro sensitization of red cells by serum antibodies or complement, the test is referred to as the indirect test.

4.2.5 Elution

a. Eluates are prepared from the red blood cells of 8 patients who have developed a positive direct antiglobulin test.

b. Five ml of blood are collected in glass test tube containing modified Alsever's solution 5 ml.

c. An equal volume of PBS is added to a volume of thrice-washed packed red cells, and the eluate is prepared by heat elution method.

d. Mix well and place in a 56°C water bath approximately 10 minutes, agitating frequently during incubation.

e. Immediately transfer the tube to pre-warmed centrifuge cups containing 56°C water.

f. Centrifuge at 3000 rpm for 5 minutes in table centrifuge

g. Remove the hemoglobin-tinted supernatant eluate, an antidrug antibody detection is done on the day of preparation or frozen at -20°C, until tested.

Principle

Elution is the removal of an antibody that has been adsorbed onto red cells either in vivo or in vitro