APPENDIX XII

Precipitating Antibody Determination

Field Operations Manual
NIOSH Contract No. 210-76-0175

<u>RATIONALE</u>

Exposure to a foreign antigen elicits the production of antibodies directed toward the antigen. If the antigen is a soluble molecule, the reaction between antibodies and antigen can be determined by precipitation reactions. The precipitated reaction is a two stage chemical which takes place in a liquid or gel matrix. The first stage the antibody reacts with antigenic determinants on the antigen. Since both the antigen and the antibody are charged molecules, the reaction is dependent on pH and ionic strength of the buffer used in the reaction. Hence, precipitation reactions are carried out in buffered media containing electrolytes. When the primary reaction has reached an equilibrium, a secondary reaction takes place. This reaction is possible since only one antigen combining site on the divalent antibody is reacted with antigen during the primary stage of antigen-antibody interaction. The second unbound antibody receptor now attaches to additional antigenic determinants on the antigen. This results in lattice formation between multiple antibodies and antigenic determinants. Visible precipitation then occurs because the lattice formation is large enough to be insoluble in the buffered media.

The precipitation reaction occurs only when there are optimal interactions between antigen and antibody. Hence, the reaction is dependent directly on the concentration of both antigen and antibody. If there is excess antibody relative to the amount of antigen in the system, visible precipitation will not be observed. Under this condition, the antibody will complex with two antigenic determinants. Because the antibody has complexed with two antigenic determinants, it is unable to react with additional antigenic determinants necessary for lattice formation. Conversely, if there is excess antigen relative to antibody in the system there will also be no visible

precipitation. The lack of visible preciptation is due to the fact that there are two antibodies to combine with all the receptor sites necessary for lattice formation.

Precipitation reactions in agarose gels are widely used in immunology to detect the presence of antibodies to soluble antigens. The reaction is identical to precipitation reactions in liquid media. Inwells are cut in solidified agarose. Antibody is placed in one well and antigen is placed in another well. During the incubation period, the antibodies and antigen diffuse toward each other and interact in a manner described previously. When optimal concentrations are achieved, lattice formation takes place and a precipitate forms that is visible through the agar as a white line.

Using immunodiffusion techniques careful consideration must be given to the size of the wells and the distance between wells. If proper sized wells are used, the optimal interactions between antigens and antibodies will not be achieved, and no visible precipitation will occur. Moreover, if the wells are too far apart for optimal interactions to occur, no visible precipitation will occur. Therefore, the immunodiffusion technique must be standardized from laboratory to laboratory to yield reproducible results.

The determination of precipitating antibodies directed against organic molecules by immunodiffusion techniques is often helpful in the diagnosis of certain lung diseases. Subjects with a history of hypersensitivity pneumonitis (HP) often have precipitating antibodies to the etiological agent in their serum (A12.1). Hence, the presence of precipitating antibodies to extracts of thermophillic actinomycetes is used to support a tentative diagnosis of HP. The presence of precipitins alone, without a clear clinical history of the disease, cannot be used to diagnose HP.

The determination of precipitating antibodies to occupation-related antigenic material is also useful determining the relative exposure of industrial

populations to agents which may cause HP. Again, the frequency of precipitins in the test population must be compared to frequency in control populations.

REAGENT

1. Panel of extracts used for determination of precipitating antibodies (Appendix X)
2. Pre-cleaned microscope slides
3. 0.1 M borate citrate buffer pH 8.4
   a. 6.19 g boric acid
   b. 9.54 g sodium tetraborate
   c. 4.38 g sodium chloride
   d. Dissolve in 1.0 liter of deionized, distilled water
   e. Add 1.0 g of citric acid
   f. Adjust the pH to 8.4
4. Agarose
5. 0.1% Agarose for precoating slides
   a. 0.1 g of agarose in 100 ml deionized distilled water
   b. Bring to a boil
   c. Cool to 56°C in a water bath
   d. Add 0.5% glycerol and stir
6. 1.0% agarose for agar slides
   a. 0.5 g of agarose in 50 ml of 0.1 M borate citrate buffer, pH 8.4
7. Coplin jars
8. Immunoframes
9. Leveling table
10. Gel cutter
11. Humid chambers

METHODS

1. Pre-coating microscope slides
   a. Place 0.1% agarose suspension in a beaker and place in a water bath on a hot plate.
   b. Bring the agarose suspension to a boil.
   c. Pour the liquid agarose into a Coplin jar large enough to immerse microscope slide.
   d. Using a pair of tweezers, quickly immerse microslides in the Coplin jar. Be sure that the entire slide is beneath surface of the liquid.
   e. Immediately remove the microscope slides and place at a 45 degree angle.
   f. Let the slides air dry.
   g. Wrap the slides in groups of six in paper towels. Be sure that the wrapping process prevents slide to slide interaction.
   h. Secure the paper with a rubber band. Mark each pack with the date.
   i. Store at room temperature.
2. Preparation of gel diffusion plates
   a. Mark six slides with the numbers 1-6 using a diamond point pen.
   b. Place the six slides in an immunoframe on a leveling table. Be sure that the slides are in numerical sequence, and that the slides are level.
   c. Weigh out 0.5 g of agarose.
   d. Suspend the agarose in 50 ml of 0.1 M borate citrate buffer pH 8.4.
   e. Place the agarose suspension in a water bath on a hot plate.
   f. Bring the mixture to a boil.

g. Let the liquid cool to 56°C in a waterbath. Keep the molten agarose in the water bath.

h. Place 10 ml of the molten agar onto the three microscope slides on one side of the immunoframe.

i. Place another 10 ml onto three slides in the other side of the immunoframe.

j. Allow the agar to harden at room temperature for 15 minutes.

k. Place the agar-coated microscope slides in a humid chamber for 30 minutes.

l. Repeat steps a-k for a second set of slides.

3. Cutting the gel diffusion wells

a. Using an LKB gel cutter, prepare two well patterns per microscope slide. Each pattern should contain six peripheral (2.0 MM in diameter) wells separated from a central well (6.0 MM in diameter by 3.0 MM).

b. Remove one immunoframe with agarose-coated slides from the humid chamber.

c. Place the well cutter over the agarose-coated slides and press the plunger.

d. Repeat the process until two patterns have been cut in each of the six microscope slides.

e. Gently remove the agarose from the wells using a capillary pipette attached to a water aspirator. Make sure that the wells are straight and free of agarose fragments.

f. Place the microscope slides in the humid chamber.

4. Immunodiffusion analyses

   a. Remove the panel of extracts used to detect precipitating antibodies from the refrigerator. Allow to warm to room temperature.

   b. Place the extracts in groups of six in test tube rack and remove the caps.

   c. Place a capillary pipette with a rubber bulb in each of test extracts.

   d. Make a master list of the extracts as they appear in each group of six.

   e. Remove the agarose-coated slides from the humid chamber.

   f. Begin with the first pattern of microscope slide #1. Add approximately 10 µl of the first six extracts to wells 1-6. Be careful not to overfill the wells. If overfilling occurs, blot with disposable wiper.

   g. Repeat the procedure with the second well pattern on the first slide using the next set of six extracts.

   h. Repeat until all of the wells are filled in all six microscope slides.

   i. Place a sample of undiluted test serum into the center well of each pattern. Make sure the well is filled (approximately 50 µl). Do not overfill.

   j. Place the slides in a humid chamber.

   k. Examine for precipitin lines at 24 and 48 hours.

<u>LIMITATIONS</u>

Although the immunodiffusion method described previously is as sensitive as other immunodiffusion methods (e.g., gel template method) and more

sensitive than the counter immunoelectrophoresis method, immunodiffusion does have several limitations. First, it is less sensitive than other methods used to detect the presence of specific antibodies. However, the more sensitive methods do not readily lend themselves to large population studies using many different antigens and test sera. Secondly, the large antibody well used in the immunodiffusion method is not commercially available and must be manufactured by the individual investigator. Third, serum lipoproteins may precipitate around the antiserum wells and inhibit the visualization of precipitin lines. Fourth, some of the precipitin lines observed in immunodiffusion method may not be classical antigen-antibody interactions. Reactions between acid proteins of the antigen can form precipitin lines in gels (A12.2). Conversely, interactions between basic proteins in serum (i.e., lysozyme) and acidic proteins in antigen preparations can also initiate non-specific precipitation in gels. Reactions of C-reactive protein, a serum complement produced during an inflammatory response, and C-polysaccharide also produce non-specific precipitation in gels. C-polysaccharide is produced by several bacteria and some species of aspergillus (A12.3). It is also conceivable that certain antigenic extracts contain a protein similar to C-reactive protein. Hence, interaction between antibodies and substances analogous to C-reactive protein initiate precipitation. Some non-specific precipitation has also been demonstrated after non-specific interaction between non-antibody serum proteins and teichoic acids of bacterial cell wall (A12.4), and serum alpha macroglobulin and certain antigens. Non-specific precipitation should be suspected in large population studies if 40-50% of the test and control populations demonstrate serological reactivity to specific antigens.

Some types of non-specific precipitation can be prevented by use of reactant modifiers or changes in the buffer system, or the use of agarose as the supporting matrix. Non-specific precipitation of serum lipoproteins can be prevented by using 1.0 M glycine in the agar gels (A12.5). The interaction of acidic-basic proteins can be prevented by the absence of NaCl in the buffer (A12.6). The use of the clotting agent citrate may prevent the CA^{++} requiring interaction between C-reactive protein and C-polysaccharide. The citrate also prevents precipitation of serum lipoproteins in agar (A12.7, A12.8).

If changes in buffers or addition of reactant modifiers fail to alter the precipitin lines, one must use other immunological methods to demonstrate the nature of the immunological reaction. To determine whether α-2 macroglobulin-antigen interactions result in precipitin lines, immunoelectrophoresis techniques can be employed (A12.9). Wells are cut on either side of a trough and test sera is placed in one well and normal serum is placed in the other well. After electrophoresis, the antigen extract is placed in the trough and allowed to diffuse toward the electrophoresed serum components. If the reaction is due to reactions between α-2 macroglobulin and antigen, a line of precipitation will be observed in the α-2 region with both the test and control sera; no line will be observed in the antibody containing α region. Conversely, if the antigen is interacting with antibodies, the line of precipitation will be observed in the γ region. The specificity of the reaction may or may not be determined by the reaction. If the reaction is non-specific, lines of precipitation will be observed in the γ electrophoretic region of both the test and control sera. If the reaction is specific, a reaction will only be observed in the γ electrophoretic region of the test serum.

It is best, however, to actually demonstrate the antigen-antibody reaction observed in immunoelectrophoresis as the result of antibody binding to antigen via the F $(ab)_2$ portion of the molecule. This can be achieved by isolating the IgG fraction of test sera by salt fractionation (A12.10, A12.11). The isolated IgG is then treated with pepsin which digests the Fc portion of the antibody but has no effect on the F $(ab)_2$ portion of the antibody (A12.12, A12.13). Since C-reactive protein antibody interactions occur in the Fc portion of the antibody, pepsin digestion, in effect, will prevent non-specific interactions from occurring via the Fc receptor. After column chromatography, to separate the F $(ab)_2$ and Fc fragments, the F $(ab)_2$ fragments are concentrated and tested in the immunodiffusion system against the same antigen. The presence of a precipitin line proves that the reaction is the consequence of classical antigen-antibody reaction since the antibody preparation lacks other serum proteins which can give false positive reactions and the reaction can only occur via the F $(ab)_2$ portion of the antibody.

<u>INTERPRETATIONS</u>

1. Presence of a precipitin line:

   The demonstration of a precipitin line indicates the previous exposure to the antigen. The presence of a precipitin line to specific antigens should be correlated with clinical history, pulmonary function changes and/or x-ray changes to determine if there is an association with lung disease.

2. Negative precipitin lines:

   The lack of a precipitin line does not preclude exposure to the antigen. Two explanations for the lack of precipitin lines can be put forth. First, the test panel may not contain the proper antigen. Second, the precipitating antibodies are present in small concentrations which cannot be detected by the immunodiffusion method.

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APPENDICES

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I. Privacy Act of 1974 - Comments

II. Research Participant's Document

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APPENDIX XIII

Determination of Immunoglobulins, C3 and α1-Antitrypsin

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RATIONALE

Several constituents of serum can be measured in vitro by immunochemical techniques. The proteins include immunoglobulins (G, A, M) complement component (C3) and $alpha_1$-antitrypsin (AAT). The usual way to quantitate these proteins is radial immunodiffusion (RID). In this technique, heterologous antibody directed toward the protein is distributed evenly within a solidified matrix. Wells are then cut into the agar and the test protein (antigen) is placed in the wells. The antigen diffuses radially from the wells into the matrix containing antibodies. When optimal concentration of antigen and antibody are attained (Appendix XII) a visible precipitate, in the form of a ring, is observed. Since the antibody concentration is fixed in the reaction, the point of optimal concentration of antigen-antibody necessary for precipitation is dependent solely on the concentration of antigen in the system. Hence, the larger the diameter of the precipitin ring the higher the concentration of antigen.

Two different radial immunodiffusion methods can be used to quantitate serum protein. In the timed technique the diameter of the precipitin ring is determined as it is still expanding (A13.1). Theoretically, the timed method is feasible since, as more antigen diffuses from the well, the precipitin ring will dissolve in antigen excess and will reappear at a more distinct point. Since the timed method requires less incubation time, it can be used when quick results are needed. The second method used to quantitate serum proteins is the limit diffusion method. In this method, the diameter of the ring is determined at the conclusion of the reaction when the precipitin ring has stopped expanding (A13.2).

The relationship of the antigen concentration to the diameter of the ring is different when the timed and limit diffusion method are compared. In the

timed reaction, an approximate linear plot is observed when the log of the antigen concentration is plotted versus ring diameter. Conversely, in the limit diffusion technique, the relationship between the diameter squared $(D)^2$ and the antigen concentration is linear.

The limit diffusion method is the method of choice for quantitation of serum proteins. This method has been found to be highly accurate and is not influenced by environmental factors (i.e. temperature changes) which may alter the results of timed tests (A13.3).

The quantitation of serum proteins is useful for several reasons. Determination of levels of IgG, IgA, IgM and C3 can be used in assessing the immunological status of test populations in cases where immunosuppression is suspected. Conversely, $alpha_1$ and antitrypsin is the major serum protein which inhibits trypsin activity in the lungs. Hence, the lack of AAT may predispose individuals to syndromes involving this trypsin-induced auto-digestion of the lungs.

REAGENTS

1. Commercially available (Calbiochem/Behring) Limit Diffusion Radial Immunodiffusion Plates for Determination of: IgG, IgA, IgM, C3, $alpha_1$ antitrypsin.
2. 0.85% saline
   a. 0.85 g of NaCl in 100 ml of deionized distilled water.
3. Test tubes
4. Microliter pipette
5. Scotch tape
6. Calibrated magnifier
7. Accuracy control for each protein
8. Internal standard
   a. Fresh frozen normal human serum

METHOD

1. RID Technique

   A. Remove the test samples and the internal control from the freezer. The method for obtaining samples is described in Appendix XIV.

   B. Allow the samples to reach room temperature.

   C. Make the appropriate dilutions of the test samples in 0.85% normal saline according to the manufacturer directions.

      1. IgG - 1:10

      2. IgA - undiluted

      3. IgM - undiluted

      4. C3 - 1:2

      5. AAT - 1:10

   D. Make a master list of the samples relating the sample to the plate and well number.

   E. Remove the RID plates, accuracy control and protein reference standards from the refrigerator. Carefully remove from the aluminum foil envelope as directed by the manufacturer.

   F. Remove the lids of the RID plates and allow them to stand open for 5 minutes at room temperature.

   G. To the first well on plates 1 and 3, add 5 µl of Standard Solution No. I.

   H. To the second well on plates 1 and 3 add 5 µl of Standard Solution No. II.

   I. To the third well on plates 1 and 3 add 5 µl of Standard Solution No. III.

   J. Add 5 µl of the accuracy control to well 4 on plates 1 and 3 and well number 1 on plate 2.

K. Add 5 µl of the internal control to well 5 on plates 1 and 3 and well number 2 on plate 3.

L. Place test serum samples (5 µl) in the remaining wells.

M. Replace the lid of the RID plate and replace in the aluminum foil envelope.

N. Seal the envelope with scotch tape to prevent loss of moisture.

2. Incubation at Room Temperature

A. IgG 50 hours

B. IgA 50 hours

C. IgM 80 hours

D. C3 48 hours

E. AAT 48 hours

3. Calibration Curves

A. Remove the plates from the foil packages.

B. Using a calibrated magnifier, measure the diameter of the precipitin rings for each standard protein solution and internal and accuracy control. The measurement must be accurate to 0.1 mm.

C. Determine the mean and standard deviation for each standard protein solution and the controls. The variability from plate to plate should be less than 0.5 mm.

D. Calculate the diameter squared for each mean value $(D)^2$.

E. Using linear graph paper plot the $(D)^2$ of the protein standards, I, II and III (ordinator) against the concentrations (abscissa). The plot should result in a straight line which intercepts the ordinate at 11 ± 3.4 mm^2. If the intercept value is greater than 14.5 mm^2 or less than 7.5 mm^2, the test must be repeated.

4. Determination of Internal Accuracy and Reproducibility

   A. Calculate the diameter squared $(D)^2$ of the accuracy control and the internal control.

   B. Determine the concentration of the controls from the protein reference calibration curve. The protein concentration of the accuracy control should be less than the standard deviation of the mean value supplied from the manufacturer. The internal control should not vary more than 2.5%.

5. Determination of Protein Test Sera

   A. Calculate the diameter squared $(D)^2$ for each test sample.

   B. Determine the protein concentration from the standard reference curve.

6. Conversion Formulae

   A. To convert mg/100 ml to I.U./ml:

      1. IgG $\frac{\text{mg/100 ml}}{100} \times 11.5 - \text{I.U./ml}$

      2. IgA $\frac{\text{mg/100 ml}}{100} \times 59.5 - \text{I.U./ml}$

      3. IgM $\frac{\text{mg/100 ml}}{100} \times 115 - \text{I.U./ml}$

REFERENCE OR NORMAL VALUES

	Mg/100 ml	International Unit/ml
IgG	800-1800	92-207
IgA	90-450	54-268
IgM Males	60-250	69-287
Females	70-280	80-322
C3	55-120	--
Alpha_1 antitrypsin	200-400	--

LIMITATIONS

There are several practical and theoretical limitations of the RID test. Both timed and end point diffusion plates are commercially available, but the end point diffusion method should be used. These plates should be prepared by the method of Mancini, Carbonara and Heremans (A13.2). When there is a question of reaction kinetics, the manufacturer should be consulted. Moreover, the RID method must be used as the manufacturer specifies. Radial immuno-diffusion plates designed for timed diffusion cannot be used to determine end point diffusion.

There are also several technical factors which may influence the results of RID. First, it is conceivable that there may be batch to batch variation in the serological reactivity of antibody in the RID plates; therefore, one must use the same lots of plates to test both test and control populations. Second, there may be some plate to plate variation in antibody reactivity in RID plates. The use of an internal standard and accuracy control on every plate can be used to detect plate to plate variation. Usually, the diameter of the accuracy control and internal standard will be 2.0 $\pm$ 1.0%. Lastly, to insure the accuracy of the standard reference curve, a three point reference protein curve should be run on every third plate.

Several sources of error can be ascribed to the filling of the wells. If the wells are not completely filled with a constant amount of antigen or the antigen is spilled outside the wells, the results will be spurious. If the wells contain air bubbles, the results will be invalid.

The determination of serum proteins by immunodiffusion technique detects only the presence of the test protein and not the functional capacity. Hence, if functional abnormalities of the serum proteins are suggested, more

sophisticated immunological tests must be employed. For example, increases in a specific immunoglobulin may suggest a monoclonal gammopathy, but clinical interpretation would depend on total serum protein levels and serum electrophoresis patterns. Conversely, decreased immunoglobulin levels may suggest an immunosuppression or immunodeficiency, but clinical interpretation will depend on assessment of the antibody mediated immune system.

INTERPRETATION

The data are difficult to interpret because certain disease conditions and/or environmental stimuli may increase or decrease the levels of serum proteins. Moreover, the levels of serum proteins may be altered by increases in synthetic rate or decreases in the metabolic rate. It is conceivable, therefore, that decreases in complement C3 may be due to an increase in catabolism rather than consumption in an antigen-antibody reaction or an immune defect in synthesis.

The AAT levels in serum present a unique problem. Decreased levels of AAT may be due to a genetically determined partial or total inhibition of synthesis of AAT. Hence, it is necessary to determine the P_i phenotype and the trypsin inhibitory capacity of (TIC) serum samples with less than 60% of the normal mean value for AAT. Phenotyping and TIC determinations and beyond the scope of most laboratories and should be done only in regional reference centers. Using these techniques the presence of the MZ or ZZ phenotype with decreased trypsin inhibitory capacity may suggest a propensity to develop emphysema. The data should, however, be evaluated in conjunction with familial and clinical history and possible exposure to agents which induce emphysema.

The following table shows the effect of certain conditions on the levels of serum proteins.

Alpha 1 Antitrypsin

Increased	Decreased
Acute/chronic inflammatory disease	familial emphysema
Stress syndrome	familial infantile cirrhosis
Malignant tumors	severe hepatic damage
Pregnancy	nephrotic syndrome
Hematologic disorders	malnutrition

Complement (C3)

Increased	Decreased
Acute inflammatory response	acute glomerulonephritis
	membranoproliferate glomerulonephritis
	immune complex disease
	active systemic lupus erythematous
	inborn C3 defect

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APPENDIX XIV

Determination of Factor B Level and Activation

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Rationale:

The complement system is a group of nine blood proteins which interact in a cascading effect. The complement cascade can be initiated by two mechanisms. In the classical pathway, antigen-antibody complex initiate the reaction, and the complement proteins interact in a defined manner (C1,42356789). The second pathway, which does not require the presence of antibody-antigen complexes, is termed the alternate pathway. The interactions of complement proteins with certain microbial or viral antigens are the results of direct interaction of C3 with the antigen (C356789). As a consequence of complement interaction by either the classical or alternate pathway, soluble complement components are liberated which can initiate the release of histamine from mast cells, initiate chemotaxis of phagocytic cells and increase phagocytosis by phagocytic cells.

It is possible to demonstrate activation of the classical or alternate complement pathway by in vitro methods. These methods are predicated on the fact that the electrophoretic mobility of intact complement components and complement fragments differ in an agarose matrix. Since complement protein C3 is necessary for both the classical and alternate pathways, the demonstration of products of C3 unique to either the classical or alternate pathway can be used to measure complement activation.

Activation of intact C3 (1C) by the classical pathway liberates four major fragments: C3a, C3b, C3c ($_1$A) and C3d (α2d). The C3d (α2d) remains in the serum. Since the C3d (α2d) fragment has a slower electrophoretic mobility than intact C3 (1) it is possible to separate intact C3 (1C) from the C3d and (α2d) in an electrical field using immunoelectrophoresis or cross-immunoelectrophoresis.

Activation of C3 by the alternate pathway liberates different complement products. Complexes of C3b, C3 proactivator convertase (Factor D) and C3 proactivator (Factor B) initiate the cleavage of C3 proactivator (Factor B) into two profactors Ba and Bb (C3 activator). The Ba fragment is quickly metabolized whereas factor Bb (C3 activator) remains in serum. Under an electrical potential, intact C3 proactivator (Factor B) migrates in the B-2 region and the Bb fragment (C3 activator) migrates in the α region. Hence, intact C3 proactivator (Factor B) and presence of the Bb fragment (C3 activator) can be ascertained by immunoelectrophoresis.

Reagents:

1. 10 ml Vacutainer tubes (EDTA or serum separation tubes)
2. Vacutainer holders
3. Tourniquets
4. Multiple sample Vacutainer needles
5. Alcohol impregnated pads
6. Sterile 2 x 2 gauze pads
7. Agarose
8. 0.1% Agarose with glycerol
   a) 0.1g of Agarose in distilled water
   b) Bring to a boil
   c) Cool to 56°C in a water bath
   d) Add 0.5% v/v glycerol and stir
9. Stock barbital buffer pH 8.6 (2x)
   a) 2.466g of barbituric acid
   b) 9.76g of sodium barbital
   c) Suspend to 1.0 liter
10. Working buffer for positive control (1x)--Buffer A
   a) Dilute 5.0 ml of stock buffer with 5.0 ml of deionized, distilled water.

11. 0.2M EDTA

    a) 76g of EDTA tetrasodium salt A

    b) 74.4g of EDTA disodium dihydrate

    c) Dissolve in 900 ml of deionized, distilled water

    d) Adjust the pH to 8.6

    e) Adjust concentration to 1.0 liter

12. Working buffer for agarose preparation and electrophoresis (1x)--Buffer B

    a) 500 ml of stock barbital buffer

    b) 400 ml of deionized distilled water

    c) 100 ml of 0.2m EDTA

13. Microscope slides 1 x 3 inch

14. Coplin jars

15. Immunoframes

16. Leveling table

17. Gel cutter and knife

18. Control sera

    a) Plasma samples recovered from blood drawn in heparin and stored at $-70^{\circ}C$

    b) Plasma samples recovered from blood drawn in EDTA and stored at $-70^{\circ}C$

19. Inulin

20. Antisera

    a) Anti-B1A/B1C

    b) Anti-C3 proactivator

21. Provials - 20 ml (Cooke Scientific)

Methods:

1. Preparation of blood samples.

   a. The arm is cleansed with alcohol impregnated pads.

   b. Venipuncture is performed without traumatizing the skin or vein using a Vacutainer and either 10.0 ml EDTA or serum separation tube.

   c. Plasma is obtained from EDTA tubes by centrifugation at room temperature for 10 minutes. Blood in serum separation tubes is allowed to clot for 5 minutes and centrifuged for 10 minutes at room temperature.

   d. Serum or plasma is recovered and aliquoted into 2.0 ml provials. Vials are labeled with subject's name or identification number.

   e. Samples are immediately stored at -70°C.

2. Precoating of microscope slides.

   a. Place 0.1% agarose suspension in a beaker and place in a water bath on a hot plate.

   b. Bring the agarose suspension to a boil.

   c. Pour the liquid agarose into a Coplin jar large enough to immerse microscope slide.

   d. Using a pair of tweezers, quickly immerse microslides in the Coplin jar. Be sure that the entire slide is beneath surface of the liquid.

   e. Immediately remove the microscope slides and place at a 45 degree angle.

   f. Let the slides air dry.

   g. Wrap the slides in groups of six in paper towels. Be sure that the wrapping process prevents slide to slide interaction.

   h. Secure the paper with a rubber band. Mark each packet with the date.

   i. Store at room temperature.

3. Preparation of gel diffusion plates.

   a. Mark six slides with the numbers 2-6 using a diamond point pen.

   b. Place the six slides in an immunoframe on a leveling table. Be sure that the slides are in numerical sequence and that the slides are level.

   c. Weigh out 0.5g of agarose.

   d. Suspend the agarose in 50 ml of the working barbital buffer (Buffer B).

   e. Place the agarose suspension in a water bath on a hot plate.

   f. Bring the mixture to a boil.

   g. Let the liquid cool to 56°C in a waterbath. Keep the molten agarose in the water bath.

   h. Place 10.1 ml of the molten agar onto three microscope slides on one side of the immunoframe.

   i. Place another 10 ml onto three slides on the other side of the immunoframe.

   j. Allow the agar to harden at room temperature for 15 minutes.

   k. Place the agar-coated microscope slides in a humid chamber for 30 minutes.

   l. Repeat steps a-k for a second set of slides.

4. Gel patterns.

   a. With a gel punch, cut two wells and a trough in the agar. The size of the wells will differ for each system and the correct size must be determined in each laboratory.

   b. Remove the plugs from the well with a capillary pipette by gentle suction using a water aspirator.

   c. Place the slides in a humid chamber.

5. Preparation of positive and negative controls.

   A. Positive Control.

      1. Weigh out 10 mg of inulin.

      2. Resuspend the inulin in 1.0 ml of working barbital buffer <u>which lacks</u> <u>EDTA</u> (Buffer A).

      3. Pipette 20 µl of the inulin suspension into a tube containing 20 µl of fresh normal human serum or normal human plasma drawn in heparin and stored at $-70^{o}C$.

      4. Incubate for 30 minutes at $37^{o}C$.

      5. Centrifuge at 1000xg for 10 minutes at room temperature.

      6. Recover supernatant fluid for use as a positive control.

   B. Negative Control.

      1. Fresh normal serum drawn in EDTA or frozen serum drawn in EDTA and stored at $-70^{o}C$.

6. Electrophoresis.

   a. Prepare 1.0 liter of 1 working barbital buffer (Buffer B).

   b. Cut filter paper strips (Reeve Angel No. 3) in strips approximately 2 inches wide and 3 inches long.

   c. Remove test serum samples from the freezer and thaw.

   d. Arrange the sera in groups of 10 samples and make a master list of the sample order.

   e. Remove the agarose-coated slides from the humid chamber.

   f. Add 4.0 µl of the positive control to the top well of slide number 1.

   g. Add 4.0 µl of the negative control to the bottom well of slide number.

   h. Add the test samples to the wells on each of the remaining slides.

i. Fill the electrophoresis chamber with the working barbital buffer solution (Buffer B).

j. Place the immunoframe in the electrophoresis chamber.

k. Place the filter paper strips on each end of the immunoframe. Let hang into the buffer vessels of the electrophoresis chamber.

7. Electrophoresis Conditions.

a. For determination of C3 activator, samples are electrophoresed at 250V. The current should be between 6-9m Amps with a running time of 75 minutes.

b. For determination of C3 activation, samples are electrophoresed at 50V for 3-6 hours. The current should be between 2-4m Amps.

8. Antiserum Placement.

a. Remove the immunoframe from the electrical field.

b. Remove the agarose from the trough with a gel knife.

c. Fill the trough with 80 µl of diluted anti-C3 proactivator or anti-C3 (B_1A/B_1C). The proper dilution of antiserum will vary depending on the source.

9. Incubation.

a. Return the immunoframes to the humid chamber and incubate at room temperature for 24-48 hours.

10. Results.

a. For determination of C3 activator, a positive reaction is denoted by a precipitin arc in the α region (Factor Bb) and a precipitin arc in the β region (C3PA). A negative reaction is denoted by a single precipitin arc in the β region.

b. For determination of C3 activation, a positive reaction is denoted by a double-humped precipitin arc in the β region.

Limitations

The determinations of complement conversion products have several limitations. The validity of the system depends on the ability of the antisera to recognize the presence of unique antigens of complement products. In the determination of C3d (α2d) this presents a problem in that antiserum to C3d (α2d) is not readily available. Antiserum to intact C3 (B1A/B1C) must be used with the assumption that the antisera will recognize the D antigen which is present on intact C3, C3b and C3c (B1A). Although the presence of C3b and C3c (1a) in the immunoelectrophoretic system does denote complement activation, it is unlikely that C3b and C3c (1A) will be demonstrated because of rapid metabolism. Therefore, the antiserum must have the capacity to recognize the D antigen. Commercially available antisera to B1A/B1C differ significantly in their ability to recognize the D antigen, and is necessary to screen several lots of antisera from several companies in order to find one which will work in the system.

Antisera used to detect activation of the alternate pathway present little problem. Antiserum to Factor B (C3 proactivator) will react with intact Factor B and the Ba and Bb fragments due to the fact that the major antigenic determinants are the Ba and Bb molecules which are present on intact Factor B (C3 proactivator) and the fragments. The most reliable source of the anti-Factor B antiserum is, however, Dr. Otto Goetze of the Scripps Institute.

In determination of activation of C3 by the classical pathway, two methods are available. Conventional immunoelectrophoresis is suitable for large population studies if adequate separation can be achieved in the electrical field. The method is less sensitive, however, than the cross-immunoelectrophoresis which detects as little at 5% conversion (A14.1, A14.2). The choice of method, therefore, depends on the individual investigator.

The demonstration of complement conversion products may be affected by technical parameters. Complement can be activated in blood samples by interactions, between plasmin and C1 or a direct effect of plasmin on C3. Hence, it is suggested that blood be drawn in EDTA, which prevents complement interactions or in serum separation tubes which also inhibit complement activation. In addition, some lots of agarose will activate complement during electrophoresis. This phenomenon, however, can be prevented by adding small amounts of EDTA to the agaroe and the buffers used in electrophoresis.

The use of agar as the electrophoretic matrix is not recommended because of electro-osmotic effect; all charged proteins will be carried to the cathode thus altering the normal electrophoretic pattern. Agarose has little electro-osmosis and no affinity for acidic or basic proteins. Hence, proteins migrate more homogeneously with greater resolution.

Interpretations

1. Activation of the classical pathway.

   Demonstration of complement fragments liberated by the classical pathway suggests that an antigen-antibody reaction has taken place.

2. Activation of the alternate pathway.

   Demonstration of complement fragments liberated by the alternate pathway suggests that complement has been activated without antigen antibody interaction.

3. Lack of complement activation by either pathway.

   The failure to demonstrate complement activation does not preclude the possibility that complement has been activated. If the reaction takes place in other areas of the body (i.e. lungs) the conversion products may be diluted to a point where they are no longer detectable by immunochemical means.

REFERENCES

Appendix 14

A14.1 Whicher, JT: Clinical Chemistry, 1978, 24:7.

A14.2 Suyehira, LS and Gewurz, H: Laboratory Medicine, 1977, 8:29.