MANUAL OF LABORATORY IMMUNOLOGY

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Lea & Febiger • 1980 • Philadelphia

Library of Congress Cataloging in Publication Data

Peacock, Julia E
Manual of laboratory immunology.

Includes index.
1. Immunodiagnosis—Laboratory manuals.
2. Immunology—Laboratory manuals. I. Tomar, Russell H., joint author. II. Title.
[DNLM: 1. Allergy and immunology—Laboratory manuals. 2. Serology—Laboratory manuals. QW525 P356m]
RB46.5.P4 1980 616.07'56 80-16716

Published in Great Britain by Henry Kimptom Publishers, London

PRINTED IN THE UNITED STATES OF AMERICA

Print Number 3 2 1

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To our colleagues—the medical technologists, students, and residents whose labors are reflected in this publication.

PREFACE

The reeling pace at which the field of immunology has grown in recent years has been reflected, sometimes rather painfully, in the clinical laboratory. Those of us who expend most of our professional energies there have struggled through masses of literature, eagerly scanning each new publication for material to fit our needs—often with success, but usually not without some disappointment. In an atmosphere of theoretical knowledge, we must effect application, while constantly studying, revising, and improvising with available reagents and materials in an attempt to meet practical needs.

This text, primarily a procedural manual, describes virtually every assay performed in our laboratory, their adaptations and variations, their strengths and weaknesses, as we and others perceive them. We cite references wherever possible to allow other laboratories access to as much additional background and pertinent information as we can provide.

We also include exhaustive, carefully referenced reviews of selected diseases, with emphasis on laboratory tools helpful in diagnosis. A section describing laboratory assessment of immunologic status includes comprehensive, referenced discussions of T and B cell surface markers, lymphocyte transformation, immunoglobulins, the complement system, and phagocytosis, as well as detailed procedures for performing related laboratory assays and for evaluating their results.

All procedures described in this manual are performed in the Clinical Immunology Laboratory, Division of Clinical Pathology, Upstate Medical Center.

References we considered most useful for additional review are indicated on reference lists by an asterisk(*).

The original manuscript of this text was placed in our laboratory and adapted for our medical technology and graduate medical programs more than a year ago. It is already covered with coffee, fingerprints, serum, and pencil marks, and with appreciation from our staff and students. May you find it as useful.

Syracuse, New York

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SPECIMEN PREPARATION

SERUM SPECIMENS

Principle. Whole blood, obtained by venipuncture, is allowed to clot, and the serum is removed for testing.

Equipment and Materials

1. Venipuncture materials: Vacuum-blood collection system, or syringe and needle.
2. Clean, dry glass tube to contain whole blood.
3. Clean, dry pour-off tube, with label.
4. Centrifuge.
5. Pasteur pipet.

Procedure

(*Note:* To prepare specimens that may contain infectious agents, see *Handling Contaminated Specimens.*)

1. Obtain whole blood by venipuncture, allowing blood to flow directly into the tube (vacuum system), or transferring it into a tube after syringe collection.
2. Allow about an hour for the clot to retract at either room or refrigerator temperature. See individual procedures (*Cryoglobulin Determination, Cold Agglutinin Assay*) for exceptions.
3. Remove the tube top, loosening the clot if it adheres and, using an applicator stick, rim the clot to prevent adhesion to the sides of the tube. Restopper tube.
4. Centrifuge for 10 minutes at approximately 900 xg..
5. Label a test tube with accession number, tests requested, and patient's name.
6. Using a Pasteur pipet, transfer the serum to the labeled tube, and dispose of the clot. Observe serum for cellular material. Repeat steps 4 to 6 if debris is noted.
7. Place a cork, or any appropriate seal, on the tube.
8. Test immediately, or place at −20°C for subsequent testing. See individual procedures (*CH_{50}, Cryoglobulin Determinations, Antinuclear Antibodies,* including *Extractable Nuclear Antibodies*) for exceptions.

REFERENCES

1. TSH-5 Standard Procedures for the Handling and Transport of Domestic Diagnostic Specimens and Etiologic Agents, National Committee for Clinical Laboratory Standards, 1978.
2. TSH-3 Standard Procedures for the Collection of Diagnostic Blood Samples by Venipuncture, National Committee for Clinical Laboratory Standards, 1979.

QUICK-FREEZING

Principle. Specimens to be frozen to −70°C are processed for rapid decrease in temperature.

Equipment and Materials

1. Dry ice.
2. 95% ethanol.
3. Plastic storage tubes with tight-fitting seals (Vanguard International).
4. Small bowl.
5. −70°C freezer.
6. Thermal gloves.
7. Filing system for identifying frozen samples.

Procedure

1. Transfer specimen to an appropriately labeled plastic storage tube.
2. Place a small block of dry ice in the bowl and crush it into smaller pieces.
3. Add 95% ethanol. (*Note:* As CO_2 gas is released, this mixture initially will "smoke.")
4. Gently swirl the specimen(s) to be frozen in the dry ice-ethanol mixture. The specimen becomes opaque as it freezes.
5. Immediately place in the −70°C freezer. (*Note:* Because of the low temperature, it is important to designate where frozen specimens are to be found. We use a card index, a freezer book, and boxes with individual holders in rows and columns, for easy identification.

INACTIVATION

Inactivation is the process that removes complement activity from a patient specimen. Complement is known to interfere with the reactions of certain syphilis tests, although the mechanism is unknown. C_{1q} can agglutinate latex particles, causing false positives in latex passive agglutination assays, such as the cryptococcal latex-antigen test, and complement may cause lysis of the indicator cells in hemagglutination assays, e.g., heterophile tests.

Body fluids are inactivated by heating them to 56°C for 30 minutes. When more than 4 hours have elapsed since inactivation, a specimen can be reinactivated by heating it to 56°C for 10 minutes.

HANDLING CONTAMINATED SPECIMENS

Principle. Specimens suspected of containing agents of communicable disease, e.g., hepatitis, are specially handled to prevent contaminating the technologist or his environment.

Equipment and Materials

1. Red adhesive tape.
2. Metal, autoclavable containers, with properly fitted covers, large enough to hold contaminated materials. Label "Contaminated" and include the suspected agent, if known. Place a small amount of disinfecting solution in the container. Autoclave before washing or disposing contaminated materials.
3. Disinfecting solution.
4. Patient sample suspected of contamination.

Procedure

1. Specimens entering the laboratory, which are suspected of being hazardous to the laboratory worker, are flagged with red adhesive tape to alert the technologists.
2. Specimen processing is carried out as indicated (see *Specimen Preparation* for the type of specimen being used), except in the following cases:
   a. All materials used in processing, as well as the original specimen container, must be disposed of in an autoclavable container clearly marked

"Contaminated." Disposable materials are separated from those to be autoclaved, washed, and returned.

b. All containers to which the specimen will be transferred should be flagged with red tape for quick identification.

c. Specimens must be tightly stoppered at all times, including during centrifugation.

3. Any testing to be done on flagged samples must be done carefully. Use gloves and discard all used materials in autoclavable containers marked "Contaminated." Any spills are absorbed by towels containing disinfectant.

4. A list of all patients known to have communicable diseases must be kept in the processing area of each laboratory department. Any patient sample confirmed for a communicable disease must be added to this list.

FICOLL-HYPAQUE; MONONUCLEAR CELL SEPARATION PROCEDURE[1]

Principle. Böyum's method is used to separate mononuclear cells from whole blood for in vitro testing.[2]

Equipment and Materials

1. Whole blood collected by the method recommended for each procedure. (See *Surface Membrane Immunoglobulin, T Cell and EAC Rosette Assays, Lymphocyte Transformation Assay.*) Blood must be kept at room temperature and used within 4 hours of collection.

2. Versenated blood for complete blood count and differential count. (*Note:* CBC and differential count need not be performed with HLA testing.)

3. Lymphocyte separation medium (LSM, Ficoll-Hypaque, Bionetics Laboratory): Specific gravity 1.076 ± .001. Store at 2 to 8°C. May be used until the expiration date noted on the label.

4. Pasteur pipets.

5. Conical, glass, centrifuge tubes, of volume sufficient to contain three times the blood volume drawn.

6. Hanks' balanced salt solution (HBSS,1×, Grand Island Biological): Store at room temperature. May be used indefinitely unless contamination occurs.

7. White blood cell pipets and hemacytometers.

Procedure

(*Note:* If cells are to be used in lymphocyte transformation assays, the following should be accomplished using sterile technique.)

1. Measure lymphocyte separation medium (LSM) into each of two centrifuge tubes to a volume equal to ½ the collected blood volume. Place in a 37°C water bath for several minutes to remove chill. Remove from bath and bring to room temperature. Allow about 30 minutes for complete warming.

2. Using Monstre pipets, dilute blood sample with an equal volume of HBSS.

3. Overlay the LSM in each tube with the diluted blood sample, taking care not to disturb the surface of the LSM. If disturbance does occur, discard tube and replace with a new tube of LSM brought to room temperature.

4. Centrifuge tubes for 30 ± 5 minutes at 400 xg.

5. Immediately remove tubes from the centrifuge. Layers should appear as shown in Figure 1-1.

6. Carefully remove the interface layer and place it in a graduated centrifuge tube.

7. Add HBSS to wash. Centrifuge at 200 xg for 15 minutes. Decant the supernate and repeat.

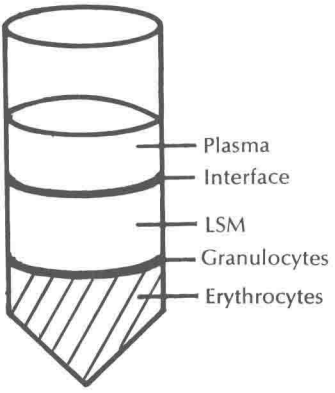

Fig. 1-1

8. Resuspend the cell button in 1 ml HBSS. (For lymphocyte transformation assays, resuspend in 1 ml RPMI-HEPES.)

9. Calculate the expected number of mononuclear cells by using the CBC and differential count results and the following equation: number of WBC × % mononuclears = absolute number of mononuclears/ml.

10. Using a white blood cell pipet and hemacytometer, count the cells in the four corner squares of the chamber and multiply by 20 for total mononuclear cells harvested per ml.

11. Compare the total cell yield per ml to the calculated absolute number of mononuclears. A yield of at least 70% is expected with LSM techniques.[4] Smaller yields may result in irregular numbers of various cell types.[3]

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DEXTRAN SEDIMENTATION; GRANULOCYTE SEPARATION PROCEDURE

Principle. Granulocytes are separated from whole blood in preparation for in vitro testing.

Equipment and Materials

1. 3% dextran: To a 100-ml volumetric flask, add 3 g dextran (MW 250,000; Sigma Chemical). Qs to 100 ml with 0.9% saline. Autoclave and store at 2 to 8°C. May be used indefinitely unless contamination occurs.

2. 3.5% saline: Add 3.5 g NaCl to a 100-ml volumetric flask and qs with distilled water. Autoclave and store at 2 to 8°C. May be used indefinitely unless contamination occurs.

3. 0.9% saline: Autoclave and store at 2 to 8°C. May be used indefinitely, unless contamination occurs.

4. Distilled water: Autoclave and store at 2 to 8°C. May be used indefinitely unless contamination occurs.

5. 0.1% heparinized saline (HS): Add 1.0 ml heparin (5000 μ/ml, Flow

laboratories) to a 1000-ml flask and qs with 0.9% sterile saline. Store at 2 to 8°C. May be used indefinitely unless contamination occurs.

6. Hanks' Balanced Salt Solution (HBSS), with calcium and magnesium ions (1×; Grand Island Biological): Store at room temperature. May be used indefinitely. Once opened, store at 2 to 8°C. Discard if contamination occurs.
7. Patient samples: Two heparin tubes (7 ml) and approximately 3 ml of blood in a clot tube are collected aseptically. Use as soon as possible after collection. (*Note:* Approximately five tubes will be needed for the *phagocytosis assay,* if mixing and matching are required.)
8. Control: Samples are obtained from a suitable control group and are handled exactly as was the patient sample.
9. Plastic tubes (40 ml) and plastic pipets.
10. Vortex.
11. Centrifuge.
12. Hemacytometer, with appropriate materials.
13. Microscope.
14. 37°C water bath.

Procedure

(*Note:* Only plastic or siliconized materials can be used since granulocytes adhere to glass.)

1. Warm reagents to room temperature.
2. In 40-ml tubes, mix the heparinized blood with approximately twice that amount of 3% dextran. Invert to mix. Remove tops.
3. Sediment for 20 minutes at room temperature.
4. Using a plastic pipet, transfer the supernate to a clean tube. Centrifuge for 10 minutes at 200 xg. Discard the supernate from this tube.
5. Lyse red blood cells as follows: (*Note:* Timing for this step is critical.)
   a. Suspend cells in 2 ml sterile 0.9% saline.
   b. Add 6 ml sterile, distilled water.
   c. Vortex for 45 seconds.
   d. Rapidly add 2 ml sterile 3.5% saline. Vortex for several seconds to mix.
   e. Centrifuge for 10 minutes at 200 xg and discard the supernate, removing as many erythrocytes as possible.
   (*N.B.:* If a significant amount of RBC remains, repeat lysis.)
6. Resuspend cells in 10 ml 0.1% heparinized saline (HS).
7. Transfer suspension to a new plastic tube to aid in removal of platelets.
8. Centrifuge at 200 xg for 10 minutes. Remove supernate.
9. Resuspend cells in 5 ml HS.
10. Count total number of cells as follows:
    a. Prepare a 1:20 dilution of each suspension using a WBC pipet. Mix and load hemacytometer chamber.
    b. Count four large outside squares.
    c. Calculate the amount of HBSS needed to give 20×10^6 cells/ml as follows: Total number of cells × .0125 = amount of HBSS.
11. Centrifuge the remaining cells at 200 xg for 10 minutes. Remove supernate.
12. Resuspend in the amount of HBSS calculated in step 10. The cell suspension is now 20×10^6 cells/ml.

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1. Goetzl, E.J., and Austen, K.F.: Immunol. Commun. *1*:421, 1972.
2. Stossel, T.P.: Blood *42*:121, 1973.

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SEROLOGIC METHODS

TECHNICAL INSTRUCTION

DILUTIONS AND SERIAL DILUTIONS; TITERS

In the clinical immunology laboratory, estimates of volume of antibody specific for a given antigen are managed by the semiquantitation technique known as "titering." Preparation of a titer involves two steps: (1) performing serial dilutions of the antibody solution (e.g., serum), and (2) adding equal volumes of antigen suspension.

Preparing a "dilution" means adding diluent so that the ratio of concentrate to total volume equals the dilution desired. Total volume equals concentrate plus diluent.

Serial dilutions represent progressive, regular increments, ranging from more concentrated to less concentrated solutions. Most commonly, serial dilutions are "two-fold"; that is, each dilution is half as concentrated as the one preceding it. When performing serial dilutions for subsequent addition of antigen solution, be sure that the total volume in each tube is the same.

The addition of antigen to a serial dilution changes the total volume in each tube, thus changing the dilution of concentrate. Unfortunately, investigators disagree over whether the final dilution reported should be considered the dilution before or after the addition of antigen or reagent.

The generation of "normal ranges" within the laboratory is necessary for a given test system. Without a normal range, titration of serum and subsequent report of an antibody dilution is meaningless.

Titers are usually reported as the reciprocal of the last dilution demonstrating the desired results; for example, when performing a two-fold serial dilution of patient serum—adding antigen suspension and observing for agglutination—the titer is reported as the reciprocal of the last tube in which agglutination was observed.

PREPARATION OF ERYTHROCYTE CELL SUSPENSIONS

Principle. Erythrocytes are washed and standardized for use in various serologic procedures.

Equipment and Materials

1. Erythrocytes: Whole human blood may be obtained in an appropriate anticoagulant, e.g., Versene. Sheep erythrocytes are obtained commercially in Alsever's solution.
2. Test tubes or conical centrifuge tubes.
3. Volumetric or Erlenmeyer flasks large enough to contain the final volume of cell suspension desired.
4. Saline, or diluent used for testing.