LECTURE NOTES

Medical Laboratory Technology Students

Serology

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In collaboration with the Ethiopia Public Health Training Initiative, The Carter Center, the Ethiopia Ministry of Health, and the Ethiopia Ministry of Education

2008

Funded under USAID Cooperative Agreement No. 663-A-00-00-0358-00.

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PREFACE

The degree, to which a laboratory service performs important function and contributes to a higher standard of health care, depends mainly on how professionals are trained. However, the scarcity of reference materials has been an impediment to the training of laboratory personnel in higher institutions in Ethiopia. Therefore, these lecture notes have been prepared to alleviate the aforementioned problems.

This lecture note presents a general introduction to serology of common infectious diseases and their serological tests. It also includes other useful serological tests. Each chapter provides learning objectives and review questions among others.

ACKNOWLEDGEMENT

We would like to acknowledge The Carter Center (EPHTI) for its financi, material and logistical support for the preparation of this teaching material. We are also indebted to Haramaya University, Faculty of Health Sciences for the realisation of this lecture note.

We extend our appreciation to the reviewers and teaching staffs of the School/Department of Medical Laboratory technology from Haramaya University, Hawasa University, Addis Ababa University and the University of Gonder for their meticulous review and constructive comments of this lecture note.

Our special thanks & gratitude goes to the external reviewers of Addis Ababa University Ato Ibrahim Ali and Ato Kassu Desta (BSc in MLT & MSc in Microbiology).

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ABBREVIATION

ACIF	Anticomplement imunofluorescense
AIDS	Acquired Immuno deficiency syndrome
ANA	Antinuclear antibodies
ASO	Anti Streptolysin O
CFT	Complement fixation test
CIE	Counter immunoelectrophoresis

CRP	C – reactive proteins
DTH	Delayed type Hypersensitivity
EBU	Epstein Barumus
ELISA	Enzyme Linked Immuno sorbentassay
ETEC	Entrotoxigenic Eschercia Coli
FACS	Fluorescent activated cellsorter
FAT	Fluorescent antibody tests
FCS	Fetal Cart Serum
FITC	Fluorescein isothyocynate
FTA-ABS	Fluorescent Treponemal Antibody absorption
HCG	Human Chronic Gondadotrophin
HIA	Hemaglutination Inhibition Antibody test
HIV	Human Immuno deficiency virus
HLA	Human leukocyte antigen
HRP	Horse reddish peroxides
IFN	Interferon
Ig	Immunoglobulin
IHA	Indirect Hemagglutination
IL	Interleukin
IMN	Infectious Mononucleosis
IRMA	Immuno radiometric assay
ISH	In situ hypridization
MNC	Mononuclear cell
NGO	Non governmental organization
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

PEG	Polyethylene Glycol
RA	Rheumatoid arthritis
RBS	Phosphate Bulter Saine
RBS	Rhosphate buffered Saline
RF	Rhumataid factor
RIA	Radio Immuno assay
RPHA	Reverse passive haemagglutination tests
RPR	Rapid plasma reagin
SLE	Systemic lapus erythematosus
SLO	Serology of Streptolysillo
SRBC	Sheep Red Blood Cells
TMB	Tetra methyl Benzidne
TNF	Tumor necrosis factor
TPHA	Treponema pallidum hemagglutination assay
TP-PA	Treponema Pallidum particle agglutination)
USR	Unheated Serum Resin
UV	Ultraviolet
VDRL	Venereal Disease Research Laboratory
WB	Western bolt
WF	Weil Felix
WHO	World Health organization

Hepatitis Virus

HAV -	Heptitis A Virus
HBV -	" B "
HCV -	" C "

HDV - " D "
HEV - " E "
HGV - " G "

CHAPTER ONE
BASIC PRINCIPLES OF IMMUNOLOGIC AND SEROLOGIC REACTIONS

Learning Objectives

At the end of this chapter, the students should be able to:

1. List different types of immunological tests
2. State the principle of immunological tests
3. Describe the application of different Immunological techniques
4. Discuss the advantages and disadvantages of different immunological techniques
5. List factors which affect antigen-antibody reaction.

1.1. INTRODUCTION

Antibody molecules combine reversibly with antigens to form immune complexes. The detection and measurements of these reactions form the basis of serology, a sub discipline of immunology.

Serology - is the science of measuring antibody or antigen in body fluids. The immune reaction is the production of antibody (substances) that protect the body against the antigen. There are times, however, when antibodies are not protective (e.g. Hay fever, rash).

Application of serology tests

Antigen tests

Antigen tests often enable an early diagnosis or presumptive diagnosis of an infectious disease through:-

- Identification of a pathogen that has been isolated by culture
- Identification of pathogens in different samples of the patients, etc

Antibody tests

These tests are used mainly:-

- To diagnose a microbial disease when the pathogen or microbial antigen is not present in routine specimen or if present is not easily isolated and identified by other available techniques.
- To screen donor blood for different infectious diseases
- To monitor the effectiveness of a given treatment by measuring antibody liter
- To diagnose autoimmune disorders, etc.

1.2 Immunological Techniques

Three groups of immunological techniques are used to detect and measure antigen- antibody reaction; these are:

- Primary binding tests
- Secondary binding tests and
- Tertiary binding tests.

1.2.1. Primary binding tests

Primary binding tests are tests that directly measure the binding of antigen and antibody (i.e.; directly measure or visualize the immune complex). They are the most sensitive techniques in terms of the amount of detectable antigen or antibody.

Example:

- Enzyme linked Immunosorbent assay (ELISA) tests
- Radioimmunoassay (RIA)
- Western blotting

Primary binding tests are performed by allowing antigen and antibody to combine and then measuring or visualizing the amount of immune complex formed. It is usual to use radioisotopes, fluorescent dyes**,** or enzymes as labels to identify one of the reactants.

1. Immunofluorescence tests

They are widely used in the serological diagnosis of bacterial, viral, fungal, and parasitic diseases. They are usually sensitive and give reproducible results.

Disadvantages of these tests are:

- Special training is needed to perform and read Immunofluorescence tests.
- Fluorescence microscope and high quality reagents are required.

Principle

Fluorescent dyes (fluorochromes) illuminated by UV lights are used to show the specific combination of an antigen with its antibody. The antigen-antibody complexes are seen fluorescing against a dark background. Immunofluorescence tests are referred to as fluorescent antibody tests (FAT).

There are two types of fluorescent antibody tests (FAT): Direct and Indirect

A. Direct fluorescent antibody tests

Direct FAT is used to detect and identify an unknown antigen in specimens, for example Viral, bacterial, and parasitic antigens. It is called direct test because the fluorescent dye is attached, or labeled, directly to the antibody. The fluorochrome used is usually fluorescein isothyocynate (FITC), which gives a yellow-green fluorescence. A fluorescent substance is one that, when absorbing light of one wavelength, emits light of another (longer) wavelength.

Procedure: Direct FAT

1. A tissue or smear containing the organism (antigen) is fixed to a glass slide and incubated with the fluorescent antibody (antibody chemically linked to FITC).
2. It is then washed to remove the unbound antibody.
3. Examined by dark-field illumination under a microscope with UV light source.
4. The antigenic particles that have bound the labeled antibodies are seen to fluoresce brightly.

Interpretation of the results:

- Presence of fluorescence: positive test for particular antigen
- No fluorescence: negative or absence of particular antigen

Direct FAT can be used;

- ✓ To identify bacteria when the numbers are very low,
- ✓ To detect viruses growing in tissue culture or tissues from infected animals such as rabies virus in the brains of infected animals or antigens of HIV on the surface of infected cells.

B. Indirect Fluorescent antibody test

In the indirect FAT, unlabelled antibody combines with antigen and the antigen antibody complex is detected by attaching a fluorescent-labeled antispecies globulin to the antibody. The antibody, therefore, is labeled indirectly. Fluorescent-labeled antihuman globulin is used if the antibody is of human origin.

The indirect FAT is used in two main ways:

- To detect and identify unknown antigen in specimens
- To detect antibodies in a patient serum using a known antigen (microorganism).

I. Indirect FAT to detect antigen

In this test, a slide preparation of the specimen is made and unlabelled specific antibody is added. After allowing time for the antigen and antibody to combine, the preparation is washed leaving only antibody that has combined with the antigen on the slide. A fluorescent labeled anti- species globulin is added and allowed to combine with the antibody. The excess is washed from the slide.The preparation is examined by fluorescence microscopy using the correct filters. The antigen-antibody complex will be seen fluorescing brightly.

II. Indirect FAT to detect Antibody

In this test, a known antigen is placed on the slide and the patient's serum is added.

The preparation is then washed and fluorescent-labeled antihuman globulin is added to demonstrate the antigen-antibody reaction. The preparation is examined by fluorescence microscope using the correct filters.

1.2.1.2. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA techniques are becoming increasingly used in the diagnosis of microbial infections. They are specific, sensitive, and require only a small amount of specimen. Reagents used in the ELISA are stable and have a long shelf life which makes for easy distribution to district laboratories. The results of qualitative ELISA techniques can be read visually. Large numbers of specimens can be tested at one time and the ELISA can be easily automated for use in epidemiological surveys. Moreover, EIA are suitable for automation, objective reading of results and the ability to link EIA plate readers to laboratory computer systems, reducing the potential for transcription errors.

Principles of Enzyme Linked Immunosorbent Assay (ELISA)

As its name suggests, the enzyme linked immunosorbent assay uses an enzyme system to show the specific combination of an antigen with its antibody.

The enzyme system consists of:

- An enzyme which is labeled or linked to a specific antibody or antigen

- A substrate which is added after the antigen antibody reaction. This substrate is acted on (usually hydrolyzed) by the enzyme attached to the antigen antibody complexes, to give a colour change. The intensity of the colour gives an indication of the amount of bound antigen or antibody.

The antigen or antibody is coated on a solid surface (solid phase) such as the inside of a plastic test tube or well of a microtitration plate, or the surface of cellulose beads. This means that after the antigen and antibody have combined, they remain firmly attached to the solid surface during the subsequent washing stage.

There are two main ways of performing ELISA

- Double antibody technique, to detect antigen
- Indirect technique, to detect and assay antibody.

A. Double antibody ELISA (Direct ELISA)

Test principle and procedure

1. Specific antibody is coated on the surface of the well of a microtitration plate (or a test tube), and the specimen is added.
2. After a period of incubation during which the antibody takes up (captures) the antigen from the specimen, the well is washed leaving the antigen attached to the antibody.

3. Enzyme labeled specific antibody (often the same antiserum as that coating the well except it is enzyme linked) is added to detect the presence of the antigen.
4. After a further period of incubation during which the enzyme labeled antibody combines with the antigen, the well is washed and a substrate is added. The enzyme acts on the substrate to give a colour change in the fluid.
5. The enzyme activity is stopped by altering the pH of the reaction or denaturing the enzyme.
6. By measuring the colour produced, the amount of attached antibody and therefore of antigen in the specimen can be estimated.

In developing countries, an important application of the double antibody ELISA is in the diagnosis of rotavirus infection in young children.

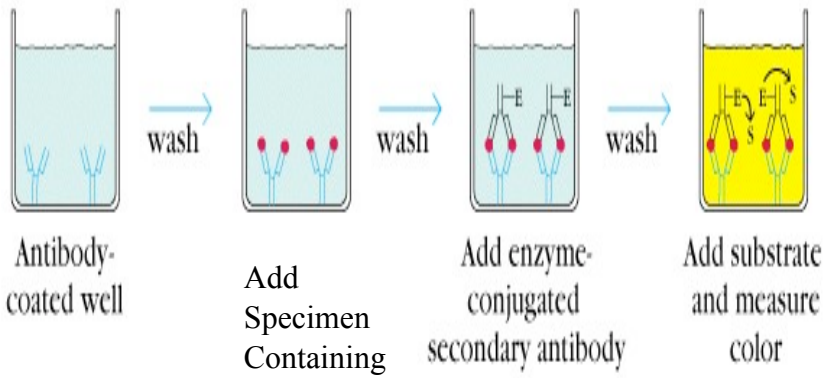


Figure 1.1 pictorial presentation of direct ELISA

B. Indirect ELISA

In this technique, known antigen is attached to the inside surface of the well and patient's serum is added. After incubation and washing, enzyme labeled antihuman globulin is reacted with the antibody that has attached to the antigen. The uncombined-labeled enzyme is washed from the well and a substrate is then added. The presence and concentration of antibody that has reacted with the antigen is shown by a change in colour of the substrate. The more intense the colour is, the higher the concentration of antibody in the serum.

The indirect ELISA is used in the diagnosis of several parasitic infections and is being increasingly used in the diagnosis of bacterial, fungal and HIV infection.

Reading ELISA results

- Many ELISA techniques, especially those that detect antigen, are qualitative and can be read by naked eye. The presence or absence of antigen is seen as a simple colour change.
- Quantitative antibody techniques are read either by measuring the intensity of colour in a spectrometer (spectrophotometer) or by testing dilutions of the test serum and determining the highest dilution that shows a colour change.

Enzymes and substrates

Enzymes used in ELISA techniques must be stable and soluble; they must not be present in any quantity in the specimens being tested. The two commonly used enzymes are horseradish peroxidase and alkaline phosphatase.

A substrate is used to give a colour change when acted on by the enzyme for example p-nitro phenol phosphate. This is hydrolyzed by alkaline phosphatase to inorganic phosphate and p-nitro phenol, which is yellow in colour.

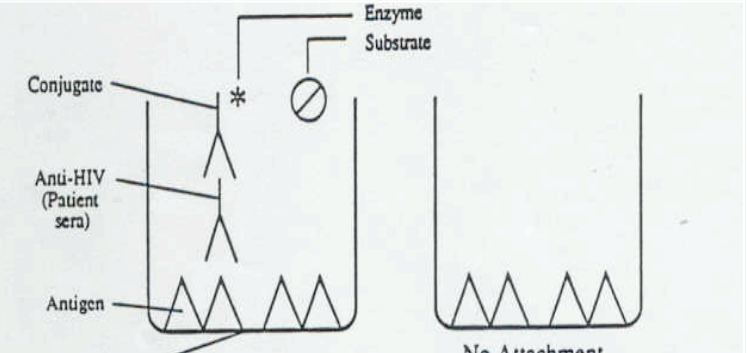


Figure 1.2 pictorial presentation of indirect ELISA

1.2.1.3. Radio Immunoassay (RIA)

A. Conventional RIA

Radioimmunoassay (RIA) is a competitive immunologic procedure for measuring very low concentrations of antigens (or antibodies) by using radioactively labeled antigens as competitors. Radioactive isotopes such as ^{3}H , ^{14}C, ^{35}S, ^{30}P or ^{125}I can be used for labeling. It is a highly sensitive method to detect low concentration of the unknown (unlabeled) antigen and is used to assay: Hormones, Drugs, Enzymes, Microbial antigens e.g. hepatitis B antigen, carcinoembryonic and α- feto proetein antigen. RIA can also be used for detection of antibody.

RIA technique utilizes three components:-

1. Patient antigen, the specific compound we wish to determine.

2. Labeled antigen, the same compound patient antigen which is attached a radioactive label.
3. Antibody, specific for the sample and labeled antigen.

Practical procedure of RIA

In a conventional RIA procedure, a defined amount of patient sample containing the antigen (sometimes called ligand) of interest is incubated simultaneously with a fixed amount of labeled antigen and a known amount of excess specific antibody. During this incubation time, labeled and unlabelled antigens compete for a limited number of antibody binding sites, usually until equilibrium is reached among sample free antigen, bound sample antigen, free labeled antigen, bound labeled antigen, and free antibody. After incubation, bound and free materials are separated from one another. By determining the amount of radioactive labeled antigen in either the bound or free fraction, the amount of patient antigen can be determined (usually the bound labeled Ag is determined)

Antibody + Antigen (serum) + ^{125}I – Antigen = Antigen-Antibody complex +
^{125}I– Antigen- Antibody complex

↓

Bound- free separation

^{125}I- antigen – antibody count in a gamma counter

In each assay, samples of known concentrations (standards or calibrators) are run to establish a standard curve for final evaluation. If the sample is infected, the amount of labeled bound will be less than in controls with uninfected serum. Since the patient antigen and labeled antigen compete for limited binding sites on the antibody molecule, the extent of binding of each depends on their relative concentrations. As these immunoassays obey the law of mass action, a small quantity of labeled Ag is bound as the quantity of sample antigen is increased and vice – versa. Therefore, the concentration of sample antigen is inversely proportional to the amount of radioactive label bound to the specific antibody. The higher the count per minute (cpm), the lower is the concentration of sample Ag and vice-versa.

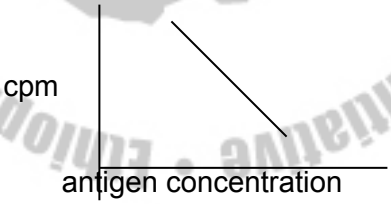


Fig.1. 3. The relationship between antigen concentration and radio isotope count per minute in conventional RIA.

Radioactive isotopes such as ^{125}I or ^{131}I (usually used for labeling antigen) disintegrate with time while emitting energy rich radiation (**γ** -rays). This radiation is absorbed by an appropriate scintillator (organic or an inorganic compound e.g. CsI, NaI) which is excited and emit visible light flash of very short duration (10 μsecond). This light flash generates electrical pulses in a photomultiplier which are further amplified and counted per unit time. This instrument set-up is a gamma-counter.

There are two assay approaches in conventional RIA;

- ✓ Liquid phase Assay
- ✓ Solid phase Assay

Liquid phase assay: In this assay principle, the sample, labeled antigen and the specific antibody are added to the mixture in a solution form. After completion of incubation with the ligand of interest (analyte), a bound- free separation step is performed using different techniques,

Solid phase assay:-In this assay, the specific antibody is added either in a suspension (with antibody bound to an insoluble particles, small magnet particles, glass beads or large carbohydrate polymers) or the antibody is covalently bound to the inside wall of the reaction tube (antibody coated tube). Separation of the bound- free fraction is realized by

centrifugation or magnetic separation followed by decanting the supernatant or by simply pouring off the reaction mixture if coated tube is used. The bound fraction is then washed adequately with appropriate buffered wash solution and made ready for counting.

B. Immunoradiometric Assay (IRMA) (Sandwich Immunoassay)

Developed with the objective of solving the problems associated with conventional RIA; which includes:-

- Physical separation methods (centrifugation decanting procedures) may distort the equilibrium between bound-free fractions, spillage loss of labeled antigen.
- Frequent non-specified binding
- Lack of sensitivity for very low concentration levels of a particular analyte.

Basic principles

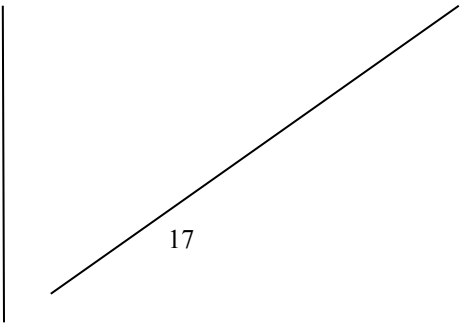
1. Specific antibody of the sample antigen is bound to the inside wall of a tube (in excess), the tube may be coated with antigen when a specific antibody is to be determined.
2. The same specific antibody is labeled with radioisotope (in antibody determinations, antigen is labeled with radioisotope)

In both cases excess labeled antibody or antigen is used

Remarks

- The antigen is specifically attached to the wall-bound antibody without competition
- As there is excess amount of bound antibody present, virtually all-available Ag molecules in the sample can be bound → increased sensitivity!
- As an excess labeled antibody is added, practically all bound Ag can be detected (effect as above)
- In most cases, the 2nd labeled antibody is specific for a different epitope of antigen molecule than the first coated antibody. This differential selectivity allows for greater specificity and enables convenient determination of high molecular weight antigens.

In contrast to RIA (conventional), in IRMA there is a direct relationship between amount of bound radioisotope and the concentration of sample antigen.



cpm

Antigen concentration in IRMA

Fig. 1.4 The relationship between antigen concentration and radio isotope count per minute in IRMA.

1.2.2. Secondary binding tests

Secondary binding tests are tests that detect and measure the consequences (secondary effect) of antigen-antibody interaction.

These consequences include:

- Precipitation of soluble antigens
- Clumping (agglutination) of particulate antigens
- Neutralization of bacteria, viruses, or toxins; and
- Activation of the complement system.

They are usually less sensitive than primary binding tests, but may be easier to perform

1.2.2.1. Agglutination Tests

In district laboratories, agglutination tests are frequently used because compared with other serological tests, they are simpler to perform, require no special equipment, and are usually less expensive.

Principle of agglutination tests

Agglutination is the visible clumping together of bacteria, cells, or particles, by an antigen combining with its specific antibody. The resulting clumps are referred to as agglutinates.

The following figure shows cells with surface antigen being agglutinated by specific antibody. This occurs when using specific antisera to identify bacterial colonies. It is also possible, for free (extracellular) antigen to agglutinate particles that have been coated with specific antibody. This occurs when testing cerebrospinal fluid for extracellular soluble antigens.

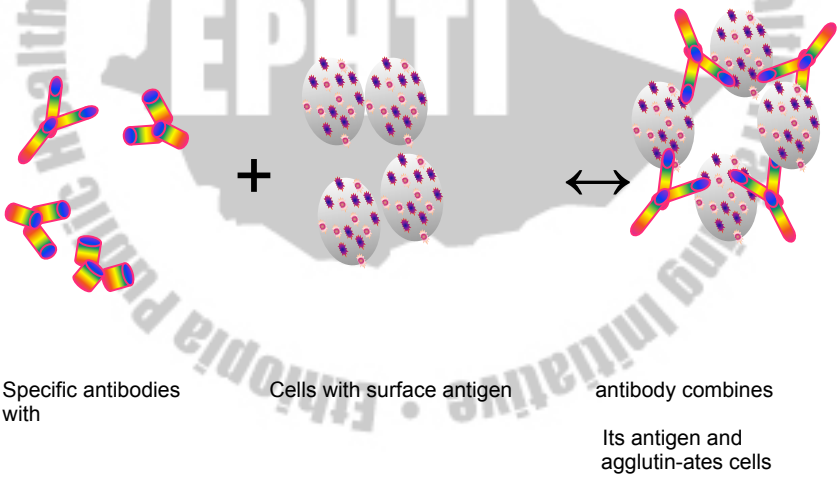


Fig.1.5 antigen antibody binding

In tests used to detect antibody **(**agglutinin) in a patient' serum, a known antigen (aggutinogen) suspension is used. The antigen particles are agglutinated if the serum contains the corresponding antibody. In general, to detect antibody in patients serum a known antigen suspension is added or to detect antigen in serum, a specific antibody is added.

Agglutination tests can be performed:

A. On slides
B. In tubes
C. In microtitration plates

A. Slide agglutination tests

These are rapid, easily performed techniques that give a reaction in minutes or even seconds. They are, however, not usually as sensitive as tube or microtitration techniques. Their specificity depends on the reagent used. The type of agglutination can be either active or passive.

I. Active agglutination slide tests

These are tests in which there is a direct agglutination of bacterial antigen with its corresponding antibody. Example, the slide agglutination of salmonellae, shigellae, or *Vibrio cholerae* by using specific antibody. Slide agglutination tests used to identify bacteria from cultures are difficult to standardize and control. False agglutination (auto- agglutination) may occur

due to the organism not emulsifying well or the fluid evaporating. It is therefore important to check first for auto-agglutination before adding the antiserum.

II. Passive agglutination slide tests

These are tests in which the specific antibody or known antigen is attached to inert particles or cells. When the known antigen or antibody combines with its corresponding antibody or antigen in the specimen, the particles or cells are used only to show that an antigen antibody reaction has occurred. Their role in the reaction is, therefore, passive.

The substances and cells used as carriers in passive slide agglutination tests include:

- Latex particles
- Carbon particles
- Stabilized staphylococcal cells

Latex particles: these are polystyrene particles that can be coated with either known antigen or specific antibody. An example of a test in which antigen coated particles are used is the antistreptolysin O (ASO) slide test. This detects significant rises of ASO antibody in the serum of patients with post-streptococcal complications. Antibody coated latex particles are used in several tests including the detection of extracellular bacterial antigens in cerebrospinal fluid.

Carbon particles: these are coated with cardiolipin antigen and used in the rapid plasma reagin (RPR) card test to screen for cardiolipin antibodies in the sera of patient with syphilis.

Stabilized staphylococcal strains: Most strains of Staphylococcus aureus produce on their outside surfaces a substance called protein A on to which specific antibody can be bound. Killed staphylococcal cells coated with antibody can be used to identify bacteria and detect soluble extracellular bacterial antigens in specimens and body fluids. The term coagglutination (COAG) is used to describe the agglutination of antibody coated staphylococcal cells by antigen.

COAG techniques are becoming increasingly used since they have been shown to be sensitive and specific for identifying important pathogens. Applications of COAG tests include, the detection of *Haemopilus influenzae* type b, meningococcal, pneumococcal, and cryptococcal extracellualr antigens in cerebrospinal fluid, the identification of pneumococcal antigen in sputum, and the detection of *Salmonella typhi* Vi antigen in urine. COAG techiques can also be used to detect *Streptococcus pyogen* (Lance field Group A Streptococcus) and other beta-haemolytic streptococci. Commercially prepared COAG reagents are expensive, but the reagents can be prepared in a microbiology laboratory at low cost (see below).

Preparation of stable COAG reagent

1. Prepare an overnight broth (protein-rich broth) culture of a protein A-containing strain of Staphylococcus aureus eg. Cowan strain or Kronwald strain.
2. Using a sterile swab, inoculate 5 plates of Mueller Hinton agar or Columbia agar with the broth culture. Use a heavy inoculum and cover the entire surface of each plate. Incubate the plates at 35-37 ^{O}C.
3. After overnight incubation, flood each plate with phosphate buffered saline (PBS) at PH 7.2. Using a glass rod, carefully suspend the colonies in the PBS taking care not to remove any agar with the colonies.
4. Using a Pasteur pipette, transfer the suspension from each plate into a centrifuge tube, and centrifuge to concentrate the organisms.
5. Wash the sedimented organisms three times in PBS, PH 7.2. Suspend the final sediment in 0.5 % v/v formaldehyde solution, stopper, and using a mixer, agitate the suspension for 3 hours.
6. Centrifuge, and wash the sedimented organisms three times in PBS, PH 7.2. Suspend the final sediment in fresh PBS, and heat to 80 ^{o}C for 1 hour.
7. Centrifuge, and wash the organisms a further three times in PBS, resuspend the sediment in the PBS, PH 7.2 to about 10 % v/v concentration.

This stabilized suspension can be stored at 4 ^{O}C for up to 6 months. The suspension must not be frozen.

8. Coat the staphylococcal cells with antibody as follows:
   - Mix 1ml of 10 % staphylococcal suspension with o.1 ml serum (see 'Note' below).
   - Mix gently for 3 hours at room temperature (20-28 ^{O}C).
9. Wash the coated cells three times in PBS, PH 7.2. Resuspend the sediment to 10ml (i.e. 1 % cells) using the phosphate buffered saline.

When stored at 4 ^{0}C, the COAG reagent will remain stable for at least 2 months. Control the reagent by testing it against known positive and negative clinical specimens.

Note: The serum used to prepare COAG reagents must be rich in lg G.

B. Tube agglutination tests

In tube tests, agglutination occurs in a larger volume of fluid and therefore, in an environment that can be more fully controlled. Tube tests are usually more sensitive than slide tests. In this tube agglutination test, serum is diluted serially and then antibody level is measured by adding standard antigenic suspension. The temperature and time of agglutination must be correct and a control tube that contains only the antigen suspension must be included to check for auto agglutination of the reagent.

In district laboratories, tube tests are used mainly to detect and measure serum antibodies in the investigation of enteric fever (Widal test), and brucellosis.

Measurement of antibody in serum (antibody titer)

To diagnose microbial diseases by their antibody response, it is usually necessary to show three or four-fold rise in the serum antibody level. This is because the patient may already have agglutinating antibodies in their serum from a previous infection, or following natural or acquired immunization. It is therefore, necessary to test two specimens (paired sera). The first collected within 5 days of the onset of symptoms and the second collected 5-10 days after the first.

The antibody level is measured by diluting the serum, usually by using a doubling dilution technique (i.e. 1 in 2, 1 in 4, 1 in 8, 1 in 16, etc). A standardized antigen suspension is then added. Following incubation for the required length of time at the correct temperature in a water bath (during which time the bacterial cells settle), the tubes are examined for agglutination. The last tube to show a clear supernatant with a coarse deposit is the end-point of the test. The dilution of the serum at this end-point is known as the titer. For example, if

the end-point is 1 in 64, the antibody titre will be recorded as 64 (1 in 64).

Prozone effect

When testing a serum with a high antibody titer, for example from a patient with acute brucellosis, it is possible for only the higher dilutions (i.e. over 1 in 40 or 1 in 80) to show agglutination. This is referred to as the prozone reaction, or phenomenon. It is thought to be due to a high level of Ig A (blocking antibody), non-specific inhibitory factors or antibody excess. Diluting the serum appropriately can solve this problem.

The following is an example of the result of an agglutination test showing prozoning:

Table 1.1 Prozone effects in antigen antibody reaction in agglutination reaction

Serum dilution	Agglutination reaction	Antigen antibody ratio
1:2	-	Prozone
1:40	-	Prozone
1:80	-	Prozone
1:160	++	Equimolar
1:320	++++	Equimolar
1:640	++++	Equimolar
1:1280	++	Equimolar

1:2560	**-**	**postzone**

Note: It is also possible when testing serum for antibodies to brucella species, for the Ig G antibodies present in the serum to combine with the antigen, but not to cause visible agglutination. The attachment of the antibody to the antigen can be detected by using antihuman globulin, which will agglutinate the antibody antigen complexes.

C. Microtitration agglutination tests

These techniques are performed in microtitration plates. They have now replaced several tube agglutination tests since they are more sensitive, more economical, easier to perform, and usually give quicker results

Types of microtitration agglutination tests

I. Indirect (passive) haemagglutination test (IHA

The indirect haemaggutination (IHA) test is a passive agglutination test (see previous text) in which known antigen is coated on treated red cells.

Carrier red cells

The cells are formalin fixed and treated with tannic acid to make the antigen adhere, Antigen coated red cells are referred to sensitized cells. In the IHA test, the sensitized red cells are added to dilutions of the patient's serum. If the serum contains the corresponding antibody in sufficient

concentration, the red cells will be agglutinated and settle to form an even covering in the bottom of the well. The antibody titer is the highest dilution of serum in which agglutination can be detected. If the sensitized cells are not agglutinated they will settle and form a red button in the bottom of the well.

IHA tests take several hours to perform (time for red cells to sediment), They require no special training or equipment. The specificity and sensitivity of the tests depend on the antigen used and how the cells are prepared. Some IHA tests give non-specific results due to heterophil antibodies present in the patient's serum. These unwanted antibodies can be removed by absorbing the serum with non-sensitized cells. Controls should include red cells of the same batch that are not sensitized with antigen to detect any antibody against the red cells. Positive and negative control sera should also be included.

Applications of IHA tests include the Treponema pallidum haemagglutination (TPHA) to detect treponemal antibodies and the antisterptolysin O (ASO) titration technique used in the diagnosis of *S.pyogens* infections.

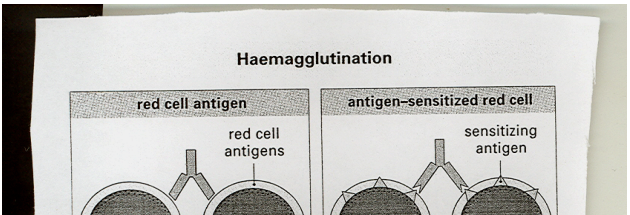


Fig. 1.6 Haemagglutination test

II. Haemagglutination inhibition antibody test (HIA)

This technique is used to detect antibodies against *Arboviruses, Ifluenza viruses*, *Measles virus*, and *Rubella virus*. These viruses are able to agglutinate red cells because they possess haemagglutinins on their outer surfaces.

In the haemagglutination inhibition (HAI) antibody test, the patient's serum is reacted with a suspension of known viral antigen. If the corresponding antibody is present, it will coat

the haemagglutinins (antigens) on the viral particles and so prevent haemagglutination when red cells are added. Controls must be included to show the agglutinating activity of the antigen and absence of haemagglutination by the serum. Sera should be treated to destroy non-specific inhibitors of agglutination. The viral antigen should be titrated.

III. Reverse passive haemagglutination test (RPHA)

This technique is used to identify viruses that do not haemagglutinate. It is performed by reacting viral specimens with red cells coated with specific viral antibody. If the corresponding antigen is present, the red cells will be agglutinated. A control must be included consisting of red cells of the same batch that are not coated with antibody.

1.2.2.2. Precipitin Tests

Precipitin techniques are used to detect and identify antigens in specimens, extracts, and cultures, and to detect and quantify antibodies in serum. Compared with agglutination tests, precipitin techniques require more experience in their performance and interpretation. Some tests have a low sensitivity.

Principle

In precipitin tests, the antigen and antibody are in a soluble form and combine to form a visible precipitate. The presence of electrolytes is usually required. Positive and negative

controls are essential. Various systems are available in which precipitation tests are performed, either in semisolid media such as agar or agarose, or nongel support medium such as cellulose acetate. Agar has been found to interfere with the migration of charged particles and has been largely replaced as an immunodiffusion medium by agarose. Agarose is a transparent, colorless, neutral gel.

The mechanisms of immune precipitation

If a suitable amount of a clear solution of soluble antigen is mixed with its antisera and incubated at 37^{o}C, the mixture becomes cloudy within a few minutes, then flocculent and within an hour or so a white precipitate settles to the bottom of the tube. This process may be analyzed by examining the effect of altering the relative proportion of antigen and antibody. If increasing amount of soluble antigen are mixed with a constant amount of antibody, the amount of precipitate formed depends on the relative proportions of the reactants. No precipitate is formed at very low antigen concentrations. As the amount of antigen added increases, a precipitate forms and increases in amount until it reaches a maximum. With the addition of even more antigen, the amount of precipitate begins to diminish, until eventually none is observed in tubes containing a large excess of antigen over antibody.

In the first of these reactions where antibody is in excess, only a little antigen is bound to antibody, free antibody may be found in the supernatant, and little precipitate is deposited. In contrast, when maximum precipitation occurs, both antigen and antibody are completely complexed, and neither can be detected in the supernatant, this is known as the equivalence zone, and the ratio of antibody to antigen is said to be in optimal proportions. When antigen is added to excess, little precipitate is formed, although soluble immune complexes are present and free antigen may be found in the supernatant. These results can be explained by the fact that antibody are bivalent and can cross-link only two epitopes at a time. But protein antigens are multivalent, since they possess many epitopes. In the mixtures containing excess antibody each antigen molecule is effectively covered with antibody which prevents crosslinkage and thus precipitation. When the reactants are in optimal proportions, the ratio of antigen to antibody cross-linking occurs. As the antigen-antibody complex grows, it becomes insoluble and eventually precipitates out of solution. In a mixture in which antigen is in excess, each antibody binds to a pair of antigen molecules. In this case, further cross linking is impossible. Hence, more complexes are small and soluble and no precipitation occurs.

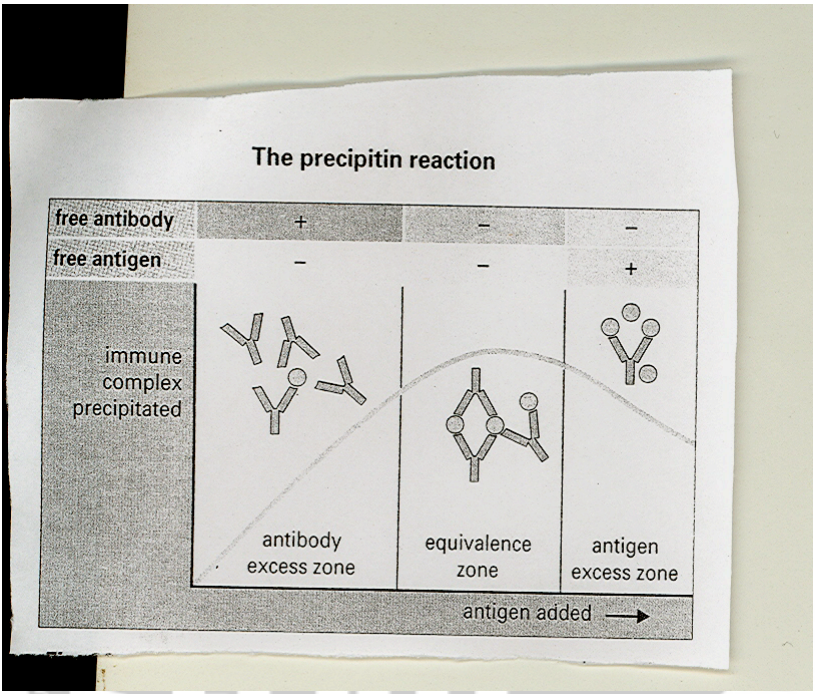


Fig. 1.7 The precipitin reaction

There are four main types of precipitin techniques. These are tube precipitin test, gel diffusion, counter immuno electrophoresis and rocket electrophoresis

A. Tube Precipitin test

In this test, a clear solution containing the test antigen is carefully layered on to a clear antiserum in a precipitin tube or capillary tube (microhaematocrit tube). Following a period of

incubation, if the corresponding antigen is present and the proportion of antigen to antibody is optimal, a line of visible precipitating antibody and antigen will form between the two layers of fluid. (Figure 1.7) A fairly large volume of antiserum is required and the test is not very sensitive. Examples of this type of technique include, the testing of cerebrospinal fluid for extracellular antigens, especially *Haemophilus influenza* type b antigen, and the original Lancefield method of grouping beta-haemolytic sterptococci in which the antigen is extracted from the streptococcal cells. Both these techniques have now been replaced in many laboratories by the simpler, more rapid, and easier to read slide tests, especially coagglutination

B. Gel diffusion

When both antigen and antibody diffuse through the agar, this is referred to as double diffusion. When only the antigen or antibody diffuses, with the corresponding antigen or antibody being contained in the agar, this is called single diffusion. Several hours of diffusion are often needed before precipitation occurs.

I. Double gel diffusion

Antigen and antibody diffuse towards each other and where they meet in optimal proportion, a visible line of precipitation forms. The thickness of the line of precipitation is a semiquantitative measure of the amounts of antigen and

antibody that combine. Double gel diffusion techniques can be used in many different ways to detect and identify antigens and antibodies. Examples of this type of reaction include, the Elek gel technique used to detect toxigenic strains of *Corynebacterium diphtheria* and the Biken test used to detect toxin-producing faecal Entrotoxigenic *Escherichia coli* (ETEC). In the Biken technique, the test and control *E.coli* organisms are inoculated in separate areas around the center of the plate. Following growth, specific antitoxin is placed in a central well cut into the culture plate. After further incubation, the toxin produced from a toxigenic E. coli strain will react with the antitoxin to form a line of precipitation, where the two meet in optimal proportion. Between the toxigenic strain and the positive control, an arc of identity will form (figure 1.8)

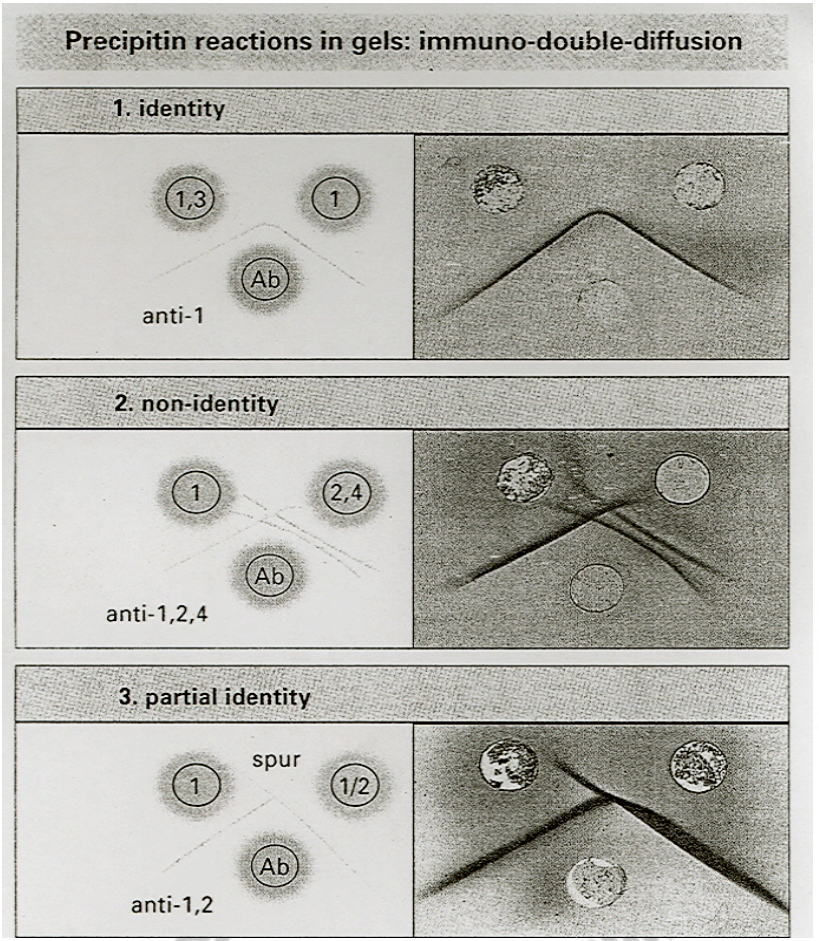


Fig. 1.8 Immuno-double-diffusion

II Single radial diffusion

In this technique, specific antibody is incorporated into the agar gel and wells are cut to contain the antigen, which diffuses radially. A ring of precipitation forms around a well that contains the corresponding antigen (figure 1.9). The higher the

concentration of antigen, the larger the ring of precipitation will be formed. By including reference samples of known concentrations, the amount of antibody in the unknown specimen can be calculated by comparing the diameter sizes of the precipitation ring.

The technique is used mainly to detect and measure immunoglobulins in serum and other specimens, for example the detection of IgM in cerebrospinal fluid when investigating trypanosomiasis meningoencephalitis. Single radial diffusion is also used to identify and quantify viral antibodies. A rapid technique is used in which red cells coated with known viral antigen and complement are incorporated in the agar gel. The test sera are placed in wells in the agar and the plate incubated at 37 ^{O}C, If a serum contains the corresponding antibody, a zone of hemolysis will form around it.

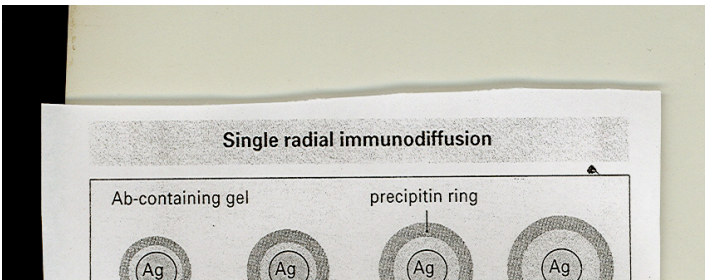


Fig. 1.9 Single radial immunoediffusion

C. Counterimmunoelectrophoresis (CIE)

This technique is also referred to as countercurrent-electrophoresis and immunoelectroosmophoresis (IEOP).

Electrophoresis is used to increase the speed with which the antigen and antibody travel in the agar gel. A line of precipitation forms where the two meet in optimal proportion. The pH, purity, and ionic strength of the agar are among the factors that influence the movement of the antibody and antigen.

Principle of Counterimmunoelectrophoresis

In this test, specific antibody is placed in a well at the positive electrode (anode) end of the plate and the unknown antigen in a well at the negative electrode (cathode) end. An electric current is applied and the antibody and antigen move towards each other. Positive samples show a line of precipitation within 30-60 minutes.

Compared with other precipitin techniques, CIE gives more rapid results, and is usually more sensitive. It has, however, the disadvantage of requiring an electrophoresis unit and the preparation of an agar gel. Counterimmunoelectrophoresis is used to detect extracellular antigens in cerebrospinal fluid.

Other application includes the detection of hepatitis B surface antigen (HbsAg) in serum.

D. Rocket electrophoresis

Antigen may be quantitated by electrophoresing them into an antibody-containing gel in the technique termed as rocket electrophoresis. The pH of the gel is chosen so that the antibodies are immobile and the antigen is negatively charged. Precipitin rockets form; the height of the rocket is proportional to antigen concentration, and unknowns are determined by interpolation from standards. Rocket electrophoresis can be reversed to estimate antibody concentration if a suitable pH gel can be found to immobilize the antigen, without damaging it or preventing the antigen-antibody reaction.

1.2.2.3 COMPLEMENT FIXATION TESTS

In general, complement fixation tests (CFT) are best performed in reference laboratories where facilities exist for the careful standardization and control of reagents, which these tests require.

Principle of CFT

The complement fixation tests is a technique that has been used over many years to detect and quantify antibody that

does not agglutinate or precipitate when reacted with its antigen, but can be demonstrated by its use, or fixation, of complement.

The complement fixation test system consists of two components:

- The first component is an indicator system consisting of a combination of sheep red blood cells, complement fixing antibody produced against the sheep red cells in another animal and exogenous source of complement, usually guinea pig serum. When these three components are combined in an optimum concentration, the antisheep cell antibody complexes and causes cell lysis.
- The second component consists of a known antigen and inactivated patient serum.

Antigen-antibody reactions lead to immune complex formation that produces complement fixation via the classical pathway. That is when complement takes part in antigen antibody reactions; it is bound or fixed to the antigen antibody complexes. When these complexes are on bacteria, red cells or other cells, the complement brings about the lysis of the cells involved. This may be exploited to determine the amount of antigen or antibody present in the patient sample. Complement fixation test can detect antibody at a level of less than one microgram per milliliter.

In the CFT, the patient's inactivated serum is serially diluted and reacted with known antigen in the presence of complement. If the corresponding antibody is contained in the serum it will combine with the antigen and use up the complement. This will leave no complement to haemolyze the antibody coated red cells that are added. The highest dilution of serum that prevents haemolysis is the antibody titre. If the patient's serum, however, does not contain the corresponding antibody, the complement will not be used and will be available to fix to and haemolyze the antibody coated red cells.

Procedure and test interpretation of CFT

The two components of the complement fixation procedure are tested in sequence:

Patient serum is first added to the known antigen, and complement is added to the solution. If the serum contains antibody to the antigen, the resulting antigen-antibody complexes will bind all of the complement (figure 1.10). Sheep red cell and hemolysin are then added. If complement has not been bound by antigen-antibody complex formed from the patient serum and known antigen, it is available to bind to the indicator system of sheep cells and hemolysin. Lysis of the indicator sheep cells indicates both a lack of antibody and negative complement fixation test. If the patient's serum does

contain a complement-fixing antibody, a positive result will be demonstrated by the lack of hemolysis.

Complement fixation tests must include controls to test:

- ✓ the stability of the red cells,
- ✓ The haemolytic activity of the complement and absence of complement binding by the antigen and by each serum tested.

Moreover, standard serum of known antibody titer must be included.

Most complement fixation tests are specific, but not always very sensitive; they give inconclusive results when the test serum contains anti-complementary substances, prozoning can also be a problem.These difficulties, combined with the considerable time it takes to perform CFT, have lead to the development of simpler techniques to replace these tests.

Complement fixation tests are still used in the diagnosis of rickettsial infections and several viral and parasitic infections.

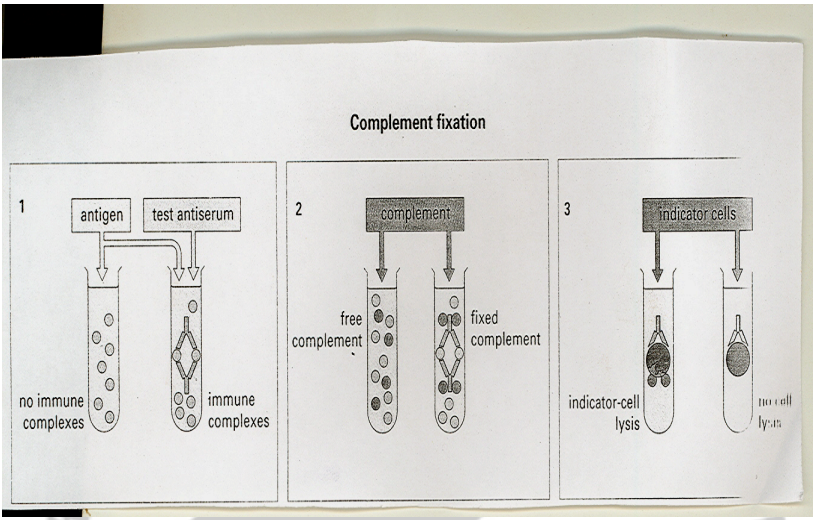


Fig. 1.10 complement fixation test

1.2.3. Tertiary binding tests

Tertiary binding tests measure the consequences of immune responses in vivo. These tests are much more complex than primary and secondary tests, but their results reflect the practical significance of the immune response. E.g. measurement of the protective effects of antibody.

1.3. Factors affecting antigen antibody reaction

Many factors affect the interaction between antigen and antibody; these include specificity, cross reactivity, temperature, pH, ionic strength, concentration, and intermolecular specificity.

Specificity: The ability of a particular antibody to combine with one antigen instead of another is referred to as specificity. This property depends on the antigen – binding fragment of an immunoglobulin molecule. Antigen – antibody reactions can show a high level of specificity.

Cross reactivity: Unrelated molecules can have antigens with similar antigenic determinants. This means a proportion of the antibodies directed against one kind of antigen will also react with the other kind of antigen. This is called cross reactivity. An example of cross reactivity is when; antibodies directed against a protein in one species may also react in a detectable manner with the homologues protein in another species.

Example of cross reactivity

Three organisms might possess antigenic structures and produce corresponding antibodies as follows:

Organism	Antigens	Antibodies
1	ABC	abc
2	BCD	bcd
3	DE	de

Antiserum prepared from organism 1 will react with organisms 1&2

Antiserum prepared from organism 2 will react with 1, 2, & 3

Antiserum produced from organism 3 will react with organism 2 & 3, but not with organism 1. Look at Fig. 1.8

Temperature: The optimum temperature needed to reach equilibrium in an antibody – antigen reaction differs for different antibodies. IgM antibodies are cold reacting, with a thermal range of 4-22^{0}C, and IgG antibodies are warm reacting with an optimum temperature of reaction of 37^{0}C.
pH: Although the optimum pH for all reactions has not been determined, a pH of 7.0 is used for routine laboratory testing.

Ionic strength: The concentration of salt in the reaction medium has an effect on antibody uptake by the membrane bound erythrocyte antigens. Sodium and chloride ions in solution have an inhibitory effect. These ions cluster around the opposite charges on antigen and antibody molecules which partially neutralizes them. This hinders the association of antibody with antigen. Reducing or lowering the ionic strength of a reaction medium, such as low-ionic strength salt, can enhance antibody uptake.

Concentration: Under normal conditions, the concentration of antigen and antibody should be optimal, but sometime this is not the case. Excess antibody or antigen concentration will result in a false reaction, sometimes known as zonal reaction when the concentration of antigen is excess it is known as a

post zone reaction; excess antibody is referred to as a prozone reaction. This phenomenon can be overcome by serial dilutions unil the optimum amount of antigen and antibody is present.

Bond strength and intermolecular attractive force

Bonding of an antigen to an antibody takes place because of the formation of multiple, reversible, intermolecular attraction between an antigen and amino acids of the binding site. The bonding of antigen to antibody is exclusively noncovalent. The attractive force of noncovalent bonds is weak compared to covalent bonds, but the formation of multiple noncovalent bonds produces considerable total – binding energy. The strength of a single antigen – antibody bond is termed as antibody affinity.

The strongest bonding develops when antigens and antibodies are close to each other and when the shapes of both the antigenic determinate and the antigen – binding site conform to each other. This complementary matching is referred to as “goodness of fit”

Review Questions

1. Define serology
2. List different groups of immunological techniques
3. Write the difference between precipitation and agglutination tests
4. Explain how different factors affect antigen – antibody reaction
5. How does the reaction of primary binding tests be visualized?
6. List different types of primary binding tests and describe their principle
7. List the application of enzyme linked Immunosorbent assay
8. Describe the advantages and disadvantages of secondary binding tests
9. How do prozone and post zone reaction affect test results?
10. List carriers used in passive slide agglutination tests and describe their applications
11. List Micro titration agglutination tests and describe their principles
12. List gel diffusion tests
13. Discuss about complement fixation test

CHAPTER TWO

SEROLOGICAL TECHNIQUES

Learning Objectives

At the end of this chapter, the students should be able to:

1. List materials and equipment for serological tests
2. Collect, preserve and prepare serological specimens
3. Run complement inactivation procedure and state its importance
4. Run serial dilution, determine end point and titer.

2.1. Introduction

Antibodies that have been produced in response to a specific stimulus can be identified easily in the serum**.** Serological reaction produces an observable change in the mixture. The reaction takes different forms, because of variations in the condition of the antigen, the presence of saline and temperature. Wide varities of serologic techniques are available to detect either an antibody or antigen using various materials and reagents.

2.2. Materials Necessary for Basic Serologic Tests

Glassware

Most Clinical Laboratories still use glassware for the greater part of the analytical work done even with the advent of modern plastic wares and stainless steel.

Certain types of glass can be attacked by reagents to such an extent the determinations done in them are not valid.

Unclean and contaminated glassware easily affect serological tests. After using, all the glassware, should be soaked in detergent for several hours and rinsed several times in clean tap water. Then allow drying in dry oven or dust free place. Scratched or broken glassware shouldn't be used, as they interfere with the reading of a test and cause damage to the skin.

The most common glassware in serological test include: test tubes, Erlenmeyer flasks, burettes, glass slide etc.

In general, serological glassware is container and volumetric apparatus.

Containers are used to contain and receive serological test solution (e.g. test tube and Erlenmeyer flask are containers whereas volumetric apparatus include pipettes and volumetric flask)

Serological pipettes

It is much like the graduated pipette in appearance. It is graduated to the end of the delivery tip and has an etched band on the suction piece (designed to be blown out).

Test tubes

Test tubes come in many sizes depending on the use for which they are intended. Tubes without lips are the most satisfactory because there is less chance of chipping and ventral breakage. Since chemical reaction (serological reactions) occurs in test tube, it should be resistant to thermal shock.

Erlenmeyer flasks

Are used commonly in the laboratory for preparing reagents for titration procedures, and for preparing blood filtrates. They also come in various sizes and must be made from a resistant form of glass.

Burettes

A burette is a long cylindrical tube of glass ware with graduation.

It is used to deliver measured quantities of fluid or solution in the process of titration. Smaller Burettes are more accurate than larger ones (they have smaller tolerances)

Glass slide

It is usually supplied with the test kit for a particular test. It must be thoroughly clean and dry before and after the test procedure. It is used in most agglutination tests.

Water Bath

A water bath is an instrument where water is heated and the set temperature is maintained at a constant level. It can provide temperature regulator and the temperature provided ranging from room temperature to 100^{O}c. Various sizes to suit various workload are available. It is used to incubate liquid substances.

Chemical tests react best at a specific temperature. Many tests react at room temperature (18 to 22 ^{o}C) and others require a specific temperature as body temperature (35 to 37 ^{o}C). Such procedural requirements are met by using water bath. When the reactants in tubes are placed in a water bath, the water surrounding the tubes warms the substances inside the tube and it takes the same temperature as the water.

Use and Care of a Water bath

1. Read the manufacturer's instructions carefully.
2. Fill the bath and maintain its level with distilled water, if unavailable with boiled water, preferably boiled and filtered rainwater. This is necessary to minimize salts depositing on the heater.
3. To minimize the growth of microorganisms in the water, add a bactericidal agent such as merthiolate at a dilution of 1 in 1000 to the water.

4. Before incubating samples, check that the temperature of the water is correct using thermometer.
5. Ensure that the level of the water is above the level of whatever is being incubated.
6. Use the lid to prevent loss of heat from the bath and to minimize particles from entering the water. When removing the lid after incubation, take care to avoid any water entering uncapped tubes. Whenever possible, use capped tubes.
7. Clean the water bath regularly, taking care not to damage the heating unit. If there is a build up of scale on heater and sides of the bath, this can be removed by using lemon juice.
8. Unplug the bath from the wall socket when not using it, when there is an electric storm, and when cleaning the bath and carrying out any maintenance work.
9. Every three to six months, check the bath for correct function.

Note: If you are using a boiling water bath and ovens, be sure that you use heat resistant glass or plastic wares.

Incubator

Incubation at controlled temperature is required for bacteriological cultures, blood transfusion, Serology, Hematology and Clinical Chemistry tests. The inside

temperature of an incubator is kept at a specific temperature (usually at $37^{O}C$). When tubes are kept inside the incubator, they take the temperature of the incubator. The appropriate temperature is obtained by means of temperature regulator and is maintained by a thermostat. This permits a more accurate temperature control.

Use and Care of Incubator

1. Read carefully the manufacturer's instruction.
2. Make sure that the incubator is positioned on a level surface and that none of the ventilation openings are blocked.
3. If the incubator does not have a temperature display, insert a thermometer in the vent hole through the roof of the incubator. Adjust the thermostat dial until the thermometer shows the correct reading, i.e., 35 - $37^{O}C$ for the routine incubation of bacteriological cultures.
4. Before incubating cultures and tests, check the temperature of the incubator.
5. Clean the incubator regularly; making sure that it is disconnected from its power supply.
6. Every three to six months check the condition of the incubator.
7. At the time of purchase, it is advisable to buy a spare thermostat and thermometer if these are of special type and are not available locally.

Centrifuge

Centrifuges are apparatus that are used to separate solid matter from a liquid suspension by means of centrifugal force. Centrifuges are used to sediment or deposit rapidly particles such as cells which may be suspended in a fluid and operated through electricity supply mainly.

Rotating machines

They are required to facilitate antigen antibody reactions. Such machines have a flat plate, and rotate at a prescribed rate of speed. A knob located on the front part of the machine controls the number of revolutions per minute.

2.3. Collection, preparation and preservation of serological specimens

Specimens that are commonly used for Serological tests include: Serum, plasma and CSF.

Blood specimens should be collected before meal to avoid the presence of chyle, an emulsion of fat globules that often appears in serum during digestion. It is ideal and necessary to use sterile dispensable blood collection system using disposable or vactuainers. Blood should be collected by vein puncture. If syringes and needles are used care must be taken to allow the blood to run gently into the clean collecting

tube to avoid rupturing of cells. The vactuainer system consists of a needle holder and glass vacuum tube instead of the syringe barrel and plunger. Once the vein is punctured, and the vactuainer tube is appropriately in contact with the needle, the required quantity of blood flows automatically into the vactuainer tube so that the need to pull the plunger out is obviated. It offers leak proof tubes and allows standardization of specimen quality. It is simple, quicker, cleaner and safer to use.

The majority of serological tests are done on serum. To prepare serum, blood is collected in a plain tube and allowed to clot completely before centrifuged. i.e., allow the whole blood to clot at room temperature for at least one hour, and then centrifuge the clotted blood for 10 minutes at 2000 rpm. Then, transfer the Serum to a labeled tube with a pipette. Serum for detection of antibodies should be drawn during the acute phase of illness or when first discovered, and again during convalescent period. A difference in antibody titer may be noted when the acute and convalescent specimens are tested concurrently. Some infections may not manifest a titer until months after the acute infection.

Occasionally, serological tests will require plasma, whole blood, urine, spinal fluid and other body fluids. Plasma

samples are obtained by treating fresh blood with an anticoagulant, then centrifuging and separating the supernatant. Cerebrospinal fluid (CSF) is collected by lumbar puncture. It is usually performed at L3 – L4 or lower to avoid damage to the spinal cord. It is usually collected by medical doctors or trained medical officers. Urine collected in clean containers at any time of the day may be used for serological tests. Morning urine generally contains the highest concentration of analyte (hormone). It should have a specific gravity of at least 1.015. If turbidity or precipitation is present, filtering or centrifugation is recommended. Urine specimen containing blood, large amounts of protein, or excessive bacterial contamination should not be used. Collect the urine specimen in a clean glass or plastic container. The urine sample may be stored at 2-8^{0}C for up to 72 hours prior to testing. Boric acid is also used as preservative for urine sample. For longer storage, the urine sample should be frozen at –20^{0}C. Thaw frozen samples by placing the frozen sample in a water bath at 37^{0}C and then mix thoroughly before use. If turbidity or precipitation is present after thawing, filtering or centrifugation is recommended. Do not refreeze urine sample.

Most serological tests should be performed within an hour after sample collection. If this could not be possible, preserve the specimen until the test is done. Serum specimen should be refrigerated at 4 – 6^{o}C and if not done with in 72 hours or

longer it should be stored at 20^0C (frozen). Serum which has been frozen may show microclots or fibrin when thawed. This should be removed by centrifugation before specimen is used. Sodium azide (1g/L) also used as preservative for blood sample.

Specimen to be frozen must be properly sealed and labeled with full patient identification. Specimen for cold agglutination must be drawn into warmed syringe & not stored in refrigerator. Care should be taken to transfer the serum to a fresh clean container. The sample should be free from hemolyzed blood as this may interfere with serological tests. Turbid sample must be centrifuged and clear supernatant must be used for testing. Therefore, specimen must not be hemolyzed and must be free from particulate matter. Contamination with alkali or acid must be avoided as these substances have a denaturing effect on serum proteins. Excessive heat and bacterial contamination should also be avoided. Heat coagulates the proteins and bacterial growth alters protein molecules. Multiple freeze - thaw cycles may result in sample deterioration. Lipemia, hemolysis or any bacterial contamination can make the specimen unacceptable. Icteric or turbid serum may give valid result for some tests, but may interfere with others.

2.4. Shipment of serological specimens

- Some times it is necessary to ship a specimen to another laboratory, a large reference laboratory, for testing.
- Tests infrequently performed or those needing specialized technology are some times more cost effective if they are done in a central or reference laboratory setting where these special tests are performed.
- Since biological specimens are potentially infectious care must be taken to ship them safely, according to the requirements established by the receiving laboratory.
- Leak proof and crush – proof primary containers and mailing containers should be used
- All specimen containers to be shipped must be labeled with the necessary patient identification, and other important information. The mailing package must include properly completed request form for the test to be done.
- Since some laboratory analyses require special handling of the specimens to be tested, transportation and handling conditions should consider the requirements of that particular test. It is always the rule to transport the specimen to the laboratory as quickly as possible, however, using the transport system

implemented by the health care institution. But do not inactivate serum / plasma before mailing.

2.5. Complement inactivation

Complement inactivation is important since it is known to interfere with different tests. Inactivation of complement can be achieved by heating (Stoichiometric inactivation of) the serum or plasma at 56^{o}C for 30 minutes. If more than four hours has elapsed since inactivation, a specimen should be re-inactivated at the same temperature for 10 minutes.

2.6. Dilution

Is the act of making weaker solution from a stronger solution. This is usually done by adding water or saline, which contains none of the material being diluted. Dilution is usually expressed as one unit of the original solution to the total number of units of final solution. Any volume of a dilution indicates the relative amounts of substances in a solution.

Serum may need to be diluted in a single or as a serial dilution if it contains a concentrated amount of antibody.

2.6.1. Serial dilutions

Serial dilution is decreasing the volume of serum progressively by maintaining a constant volume of fluid. Most commonly serial dilutions are two fold that is each dilution is half as

concentrated as the preceding one. The total volume in each tube is the same. Occasionally, the first dilution may be different from the rest. The first dilution determines the starting point and the remaining dilutions determine the magnitude of the increase.

Table1.2 An example of two fold serial dilution

Tube number	1	2	3	4	5
Saline(ml)	1	1	1	1	1
P a t i e n t serum(ml)	1	1 of 1:2	1 of 1:4	1 of 1:8	1of 1:16
Final dilution	1:2	1:4	1:8	1:16	1:32

A general rule for calculating the concentration of solutions (Patient serum in each tube) obtained by dilution in series is to multiply the original concentration by the first dilution (expressed as a fraction), this by the second dilution and so on until the desired concentration is known.

2.6.2. Determination of end point and titer

In the above example, after serially diluting the patient serum, equal amount of an antigen is added to each dilution to observe the immunologic reaction. The last tube that shows a visible immunologic reaction is known as the end point of the

test, the dilution of antiserum (antibody) at the end point is known as the titer. The reciprocal of the greatest reacting dilution of the serum is considered as the measure of titer or the concentration of antibody. For example, if the highest dilution of the serum that shows a visible reaction is a 1:32 dilution, the titer of the test is expressed as 32.

Review Question

1. List the possible serological specimens
2. Explain how serum and plasma are collected, prepared and preserved for serologic tests
3. Disscus how complements are inactivated for serological tests
4. Define dilution and serial dilution
5. Explain end point and titer

CHAPTER THREE
COMMON SEROLOGIC TESTS FOR BACTERIAL AND PARASITIC INFECTIONS

Learning Objectives

At the end of this chapter, the students should be able to:

1. Describe and /or perform serologic tests of
   - Bacterial infections. (syphilis, typhoid fever, paratyphoid fever, typhus),
   - Antistreptolysin O.
   - Parasitic infection (Toxoplasmosis, hydated disease).
2. List factors affecting each serologic test
3. Explain how to read, interpret and report serologic tests.

3.1. Syphilis serology

3.1.1. Introduction

Syphilis is a chronic systemic infectious disease. Syphilitic infection may be divided into congenital and acquired infection. Acquired syphilis is transmitted by direct contact with infectious exudates from early lesions of skin and mucous

membranes of infected persons during sexual contact. Syphilis can also be transmitted through blood and blood products. Congenital syphilis is transmitted through transplacental infection or at delivery by having contact with maternal lesion.

The causative agent is *Treponema pallidum,* subspecies pallidum, a spirochetes that has never been cultured successfully on artificial culture media and it does not take up Gram's stain.

Three other treponemes (subspecies *T. pertenue, T. endemicum, and T. carateum*) are pathogenic for human. Infection with these organisms will cause serologic tests for syphilis to be reactive, although not sexually transmitted.

3.1.2. The stages of acquired syphilis

A typical case of acquired syphilis progresses as follows:-

A. **Incubation stage:** Over a period of 2 to 6 weeks after entering the body, the organism multiplies and spreads through out the body.

B. **Primary stage:** An inflammatory response at the original entry site causes formation of a chancre, a hard, painless non-discharging lesion.

   It develops after 10 days to 3 months, usually 3 weeks after infection

One or more primary chancres usually develop on the genitals, but can develop on lips or hands.

Treponemes are detectable in specimens from these lesions using dark-field microscope.

Serum antibodies can be detected 1-4 weeks after the chancre has formed

Primary lesion heals spontaneously over a period of 2-5 weeks.

C. **Primary latent period:** All external signs of the disease disappear (6 weeks to 6 months), but blood tests diagnostics for syphilis are positive.

D. **Secondary stage:** symptoms appear, disappear, and reappear over a period of about five years, during which the patient is contagious.

These symptoms include rash, skin eruption, mucouspatches (tongue, cheeks, and gums). Hence, kissing spreads the infection. These lesions are microscopically positive and all serological tests are reactive. Secondary lesions heal spontaneously over a period of 2-6 weeks.

E. Secondary latent stage

- Is asymptomatic
- Can last for years or even a life time

- Late latent stage, the reactivity in non treponemal tests decreases over time
- In some patients syphilis does not progress beyond this stage, but in many patients it progresses to the tertiary stage.

F. Tertiary stage (Late syphilis)

- Some times around 20 years after initial infection, 30% of patients with untreated latent syphilis progress to late syphilis
- Is a slowly progressive inflammatory stage in which granulomatous lesions (gummas) develop in skin, bones, stomach and other organs, and degenerative changes occur in the central nervous system causing meningovascular syphilis and general paralysis.

Moreover, cardiovascular syphilis can cause problem at aorta.

- Treponemes are not present in late stage syphilitic lesions.
- About 30% of patients with late syphilis show non-reactive non treponemal tests
- Treponemal tests are almost always positive.

3.1.3. Congenital syphilis

Congenital syphilis is acquired during fetal life from the maternal circulation through the placental passage of *T. pallidum* from the 18th week of gestation on ward or at delivery by having contact with maternal lesion. This is more likely to occur when the mother is suffering from early syphilis, particularly the primary and secondary stage rather than late syphilis. Treatment before 18th week of pregnancy prevents the infection. The clinical manifestation of congenital syphilis may be divided in to early, late and the stigmata. Many of the lesions of the first two years of life are infectious and the late lesions from third year onward are of gummatous type.

3.1.4. Immunologic Manifestations

In the Treponemes, two classes of antigens have been recognized. Those restricted to one or a few species, and those shared by many different spirochetes. Specific and non-specific antibodies (non-treponemal antibodies) are produced in the immunocompetent host against these antigens.

Nontreponemal antibodies often called reagin antibodies are produced by infected patients against components of their own or mammalian cells (i.e. against the cardiolipin or lipoidal antigen) as well as to lipoprotein like material released from the treponemes.The antilipoidal antibodies are antibodies that are produced not only as a cosquence of syphilis and other treponemal disease, but also in response to non trepnemal disease of an acute or chronic nature in which tissue damage

occurs (e.g. measles, chicken pox, hepatitis, infectious mononucleosis, leprosy, tuberculosis, leptospirosis, malaria, rickettsial disease, trypanosomiasis, and lymphogranulomavenerum).

3.1.5. Diagnostic Evaluation

The diagnosis of syphilis depends on clinical skill, demonstration of microorganism in a lesion (dark field microscopy), and serologic testing. Polymerase chain reaction (PCR) testing may be employed if available.

3.1.5.1. Darkfield Microscopy

This allows rapid demonstration of spirochaetes from primary chancres, mucous membrane lesions of secondary syphilis or congenital syphilis. The lesion is cleaned with saline, squeezed gently, and a drop of expressed exudates placed onto a drop of saline on a glass slide. If dark-field microscopy is immediately available, motile treponemes can be seen directly in the wet preparation. If dark-field microscopy is not available, an alternative technique is to allow the sample to dry and to send the slide to a laboratory for detection of treponemes by an immunofluorescent technique by using fluorescein-labeled antitreponemal antibodies. Materials collected from oral lesions should not be examined as non pathogenic oral spirochetes can contaminate the specimen.

Principe of Darkfield microscopy

The darkfield microscope is designed to eliminate the need for staining and to achieve contrast between the organism and the background. The condenser lens of the microscope does not permit light to be transmitted directly through the specimen and into the objective lens. The condenser lens focuses light on the specimen at an oblique angle, such that light that does not reflect off an object does not enter the objective lens. Therefore, only the light that reflects off the specimen will be seen and the light simply passing through the slide will not enter the objective. The field will appear dark; spirochetes viewed with a dark field microscope appear very bright on a dark background.

3.1.5.2. Serologic Tests

Most syphilis is diagnosed on the basis of serology. Serology may be positive at the time of presentation of most primary chancres (e.g. in approximately 75% of cases). However, negative serology in a patient with a genital ulcer does not exclude syphilitic chancre. Classic serologic methods for syphilis measure the presence of two types of antibodies: treponemal and non-treponemal antibodies. Those, which measure the presence of non treponemal antibodies are called non-treponemal tests, while those that measure the treponemal antibodies are called treponemal tests.

A. Standard Nontreponemal Tests

All of the current non-treponemal procedures for syphilis are flocculation tests using cardiolipin, lecithin and cholesterol as an antigen. All non-treponemal tests are performed in a similar manner: after human serum or plasma is mixed with the antigen and rotated for a few minutes, the flocculation (suspended antigen-antibody complex) can be observed. The reaction can be read by naked eye in macroscopic tests or by using microscope in microscopic tests.

The Venereal Disease Research Laboratory (VDRL) test and the rapid plasma reagin (RPR) test are the most commonly used nontrpeonemal tests. Only the VDRL and RPR tests are suitable for quantitative evaluations. Any specimen found reactive in a nontreponemal test should have treponemal tests performed before a diagnosis is made. A reactive RPR or VDRL test should be titrated in serial dilution and reported as reactive at the highest dilution which gives a reactive result. Where a diagnosis of syphilis is confirmed, the titer gives a baseline from which change can be measured: falling titers should follow successful treatment, and a four-fold or greater rise in titer is indicative of reinfection. Persistence of nontreponemal antibody in moderate or high titer usually reflects continuing infection.

There are a number of limitations associated with nontreponemal tests. Firstly, they lack sensitivity in late stage infection: 30% of patients with late latent or late active syphilis will show a non-reactive result. Secondly, 1-2% of patients with secondary syphilis exhibit a prozone reaction. Prozone occurs when an excess of antibody in undiluted serum inhibits flocculation with the antigen, giving rise to weakly reactive, atypical or occasionally false negative results. Finally, antibodies detected by nontreponemal tests are not only produced as a consequence of treponemal infection, but also in response to other conditions where tissue damage occurs.

I. Microscopic Methods

1. Venereal Disease Research Laboratory (VDRL) Slide Test

In the VDRL test, heat-inactivated serum (to inactivate complement) is reacted with freshly prepared cardiolipin-cholesterol - lecithin antigen and the resulting flocculation is read microscopically using 10x objective and 10x eyepiece. Reactive tests are quantified to obtain the antibody titer (double dilution is used).

N.B.

- Cardiolipin is extracted from beef heart tissue and to make cardiolipin antigen, it is complexed with cholesterol and lecithin (to produce standard reactivity in tests)

- In VDRL, the antigen is not stabilized and a suspension must be freshly prepared on the day of use.

The advantage of the VDRL test:

- It can be performed on serum or cerebrospinal fluid (CSF).

The disadvantages of the VDRL test:

- The antigen must be prepared fresh daily
- The test needs to be read with the aid of a microscope, and
- Serum specimens must be heat inactivated before testing

Result reporting

- Specimens exhibiting medium and /or large flocculation particles are reported as reactive.
- Those with small particles are reported as weakly reactive
- While those with complete dispersion of antigen particles or slight roughness are reported as non-reactive.
- Sera exhibiting slight roughness should be quantitated to check for the prozone phenomenon.

Quality control

Run positive and negative controls with patient sample.

2. Unheated Serum Reagin (USR) Test

- It is an improved version of the VDRL test performed on unheated serum and using a stabilized antigen.
- In all other aspects, the test is performed and read like the VDRL slide test.

II. Macroscopic Methods

1. Rapid Plasma Reagin (RPR) Test

Rapid plasma reagin is the most popular non-treponemal test. In the RPR test, the cardiolipin cholesterol lecithin antigen has added to it choline chloride. The addition of choline chloride removes the need for heat inactivation of samples and enables plasma as well as serum to be used in the test. It also enhances the reactivity of the antigen.

The antigen is supplied in a ready to use stabilized (EDTA is used) form which can be kept for up to 6 months when stored at 4-10 ^{0}C. The RPR card test antigen also contains charcoal (carbon) to make the reaction visible (macroscopic) to the naked eye (carbon particles become trapped in the floccules). The disadvantage of the RPR test is that it cannot be used for testing CSF specimens because of a lack of both sensitivity (40%) and specificity (85%).

Procedure of RPR test

In the RPR test the patient's serum or plasma is spread within a marked circular area on plastic coated card, antigen is added, and the mixture rotated at 100 revolutions per minute (rpm) for 8 minutes using a mechanical rotator. When a reactive serum is tested, the charcoal particles are entrapped in the antigen-antibody aggregates and show up as black clumps against a white plastic coated card. In the absence of antibody, the test mixture is uniformly grey. Reactive tests are quantitated to obtain antibody titer.

Result reporting

Specimens exhibiting medium to large flocculation are reported as reactive.

Specimens with an even dispersion of antigen particles or specimens that are slightly rough are reported as non-reactive.

Quality control

Run positive and negative controls with patient sample.

2. Toluidine Red Unheated Serum Test (TRUST)

This test is a minor modification of the RPR. Rapid plasma reagin test uses a lipid soluble black dye (charcoal), while TRUST uses toluidine red instead of charcoal to visualize the reaction. Except for this difference, all aspects of these tests are similar.

Note:

The serologic tests for syphilis are divided into screening tests and confirmatory tests, based on the specific antigen used.

Non treponemal (reagin) tests may be used either as qualitative or quantitative tests.

- Qualitative non treponemal tests are frequently used as screening tests to measure IgM and IgG antibodies to lipoidal materials released from damaged host cell, as well as to lipoprotein like material released from the treponemes.
- The determination of non- treponemal serum titers in a quantitative test may be helpful for more correct interpretation of results and for evaluation of patients after treatment.

In primary syphilis, reactivity in these tests does not develop until 1-4 weeks after the chancre first appears. For this reason, patients with suspected lesions and non-reactive nontrpeonemal test should have repeat tests performed at 1-week, 1month, and 3 months intervals from the time of initial testing. Non-reactive tests during the 3-months period exclude the diagnosis of syphilis.

The Nontreponemal tests are reactive in secondary syphilis almost without exception, and usually in titers of 16 or greater

regardless of the test method. Less than 2% of sera will exhibit a prozone reaction.

Nontreponemal test titers in early latent syphilis are similar to those of secondary syphilis. However, as the duration of the latent stage increases the titer decreases. Since these tests detect antibodies against a non-specific antigen shared by treponemes and mammalian tissues, a positive result is sometimes obtained with sera from healthy individuals or patients without clinical evidence of syphilis. These reactions are termed Biological False Positive (BFP). Tests using specific *T. pallidum* antigen are required to distinguish BFP reactions and treponemal infection.

B. Treponemal tests.

Treponemal tests are based on the detection of antibodies formed specifically to the antigenic determinants of the treponemes. Anti-treponemal antibodies may be detected by a variety of techniques based on agglutination, EIA, immunofluorescence, immunoblotting, and Immunochromatography. All treponemal tests use *Treponema pallidum* or its components as the antigen.

In contrast to the Nontreponemal tests, the treponemal tests should be reserved for confirmatory testing when the clinical sign and /or history disagree with the reactive Nontreponemal test results.

Like the Nontreponemal tests, treponemal tests are almost always reactive in secondary and latent syphilis. For most cases, once the treponemal tests are reactive, they remain so for the patient's life time. In fact in some patients with late syphilis, a reactive treponemal test may be the only means of confirming the suspected diagnosis. Currently, none of the treponemal tests are recommended for use with CSF.

There are different test procedures which include Fluorescent Treponemal Antibody absorption (FTA-ABS) test, *T.pallidum* Hemagglutination Assay (TPHA), *T. pallidum* particle agglutination test (TP-PA), EIA, etc.

I. Fluorescent Treponemal Antibody Absorption (FTA - ABS)

It is the most sensitive of all syphilis tests, but it is technically the most difficult. Both the performance of the test and the reading of results have to be thoroughly checked.

The FTA-ABS uses a killed suspension of *T. pallidum* spirochetes as the antigen. This procedure is performed by over laying whole treponemes fixed to a slide with serum from patient's suspected of having syphilis because of previously positive VDRL or RPR test.

The patient's serum is first absorbed with non-*T. pallidum* treponemal antigen to reduce non specific cross-reactivity.

Fluorescein- conjugated antihuman antibody reagent is then applied as a marker for specific antitreponemal antibodies in the patients' serum. FTA-ABS is the first serological test to become positive following infections i.e. 3-4 weeks after infection.

II. The Treponema Pallidum Hemagglutination (TPHA) Test

Is also extremely sensitive and has the advantage of being easy to perform.

Principle

- In the TPHA test, patient's diluted serum samples are mixed in the wells of a microtiration plate with sheep or avian red cells coated (sensitized) with T. pallidum antigen. Un-sensitized cells added to a second well serve as a control.
- If antibody is present, the sensitized cells are agglutinated and they settle in a characteristic mat pattern in the bottom of the well.
- Un-agglutinated cells in a negative test and control well form a button or smooth ring at the bottom of the well.

Reading result

Results for the TPHA are reported as reactive (1+, 2+, 3+, 4+,) or non-reactive (±, -). Completely negative readings vary in

pattern from a solid compact button of cell to a circle of cells with small central hole.

III. *Treponema Pallidum* Particle Agglutination (TPPA) Test

The most recent modification of TPHA is the use of gelatin or polymer particles, rather than erythrocytes, as carrier for the *T. pallidum* antigen. The Treponema pallidum particle agglutination (TPPA) test, for example, uses coloured gelatin particles as antigen carrier and can be performed on serum or plasma.

The use of gelatin particles has almost eliminated non-specific agglutination reactions and it is claimed that the sensitivity of the test in primary syphilis has increased due to the improved IgM binding capacity of the sensitized gel particles. As a consequence, many laboratories have replaced TPHA with TPPA.

Result interpretation and quality control are similar to TPHA.

IV. Enzyme Immunoassay (EIA)

The use of the enzyme immunoassay technique for the detection of treponemal antibodies was first described in 1975. Indirect, competitive and capture EIAs for the detection of anti-

treponemal IgG, IgM or IgG and IgM have been developed and numerous commercial kits are available.

The advantages of EIA are suitability for automation, objective reading of results and the ability to link EIA plate readers to laboratory computer systems, reducing the potential for transcription errors. Recent studies suggest that certain new recombinant antigen-based EIAs are now among the most sensitive and specific treponemal tests.

V. Immunoblotting

Immunoblotting allows for the detection of antibodies to individual proteins. In the Treponemal Western blot, solubilized *T. pallidum* proteins are separated by gel-electrophoresis according to their molecular size. The separated proteins are then transferred onto a nitrocellulose membrane which is dried and cut into strips. After incubating the strips with patient's serum, antigen-antibody complexes are visualized by adding enzyme-conjugated anti-human globulin followed by substrate, which causes a colour reaction. It is generally agreed that detection of antibodies to immunodeterminants with molecular masses of 15, 17, 44.5 and 47kd are diagnostic for acquired syphilis. Studies suggest that the assay (using IgG conjugate) is more sensitive and specific than the FTA-ABS test.

A recent development is the use of recombinant antigens instead of fractionated proteins. For example, the INNO-LIA Syphilis test (Innogenetics) is a line immunoassay utilizing three recombinant antigens and one synthetic peptide derived from *T.pallidum* proteins. The four specific antigens are coated as discrete lines on a nylon membrane with plastic backing and the test is performed as described earlier.

VI. Immunochromatographic card test

A drop of whole blood, serum or plasma is placed on the card and as the sample migrates through the card, it reconstitutes and mixes with selenium-conjugated *T.pallidum*. The mixture then continues to migrate to the “patient window” site, which contains immobilized *T.pallidum* antigen. If antibodies to *T.pallidum* are present in the sample, they bind to the conjugated antigen and to the immobilized antigen, forming a red line at the patient window site. The test can be read after a minimum of 15 minutes up to 24 hours.

Generally, all the treponemal tests described so far detect anti-treponemal IgM, IgG or IgG and IgM, and once reactive usually remain reactive for life unless treatment is given early in infection. They, therefore, indicate that the patient has had a treponemal infection at some time but cannot distinguish

between past and present infection. More over, they can not be used to monitor response to treatment.

VII. Treponemal tests for specific IgM

Detection of anti-treponemal IgM is useful in the diagnosis of neonatal congenital syphilis because IgM does not cross the placenta and therefore, cannot be maternal derived. Its presence also suggests active disease in adults who have no history of recent treatment.

Today, most laboratories that test for treponema-specific IgM use an IgM EIA, particularly IgM capture EIA. In this technique, anti-human IgM is fixed to the wells of a microtitration plate. These fixed antibodies indiscriminately "capture" IgM present in the serum. Any bound IgM which is *Treponema* specific is then detected by an indicator system, which incorporates a treponemal antigen labeled with an enzyme. In symptomatic congenital syphilis the IgM capture EIA has a sensitivity of between 88-92% and a specificity of 95%**.**

VIII. Polymerase Chain Reaction Testing for Treponema pallidum

Polymerase Chain Reaction testing of a swab taken from a genital ulcer or mucous membrane lesion (and sent to the

laboratory in a dry tube) is becoming increasingly available and has good sensitivity and specificity.

3.2. Agglutination Tests for Febrile Diseases

3.2.1. Salmonella

This genus consists of motile non-lactose fermenting, gram negative bacilli which are parasites of the intestinal tract of man and animals, including birds.

In their distribution and pathogencity, they fall into two groups:

1. *S. typhi and S. paratyphi* A, B and C are human pathogens, though *S. typhi* and *S. paratyphi* B has been isolated from bats and other animals respectively. The organisms almost always enter via the oral route, usually with contaminated drink or food. Salmonella causes three main types of disease in humans, but mixed forms are frequent. These diseases are:
    a. The enteric fevers; Typhoid, paratyphoid, and non typhoid fevers caused by *S. typhi, S. paratyphi* A and *S. paratyphi* B, and *S. scholtmuelleri* respectively.
    b. Bacteremia: is commonly associated with *S. choleraesusis*
    c. Enterocolitis: *S. typhimurium* is the prominent cause, but can be caused by any types of salmonellae.

2. All other salmonellae are primarily animal pathogens which occasionally cause disease in man.

Subdivisions

Biochemical reactions serve to define the group as a whole and aid in the differentiation of a few species which have special peculiarities. Classification mainly depends on antigenic composition. In the Kauffmann- white scheme, the salmonellae are divided into groups on the basis of their O or somatic antigens, within each group "species" are differentiated on the basis of their H or flagella antigens. About 2000 "species" i.e. different serotypes can be recognized in this way. Some species possess additional Vi (virulence) antigens.

The O groups first defined were designated by capital letters A to Z and those discovered later by the number (51 – 67) of the characteristic O antigen.

- Group A- E contains nearly all the salmonellae that are important pathogens in man and animals.

Note: Agglutination tests with absorbed antisera for different O and H antigens form the basis for serologic classification of the salmonellae.

- **O antigens:** are proteins- polysaccharide- Lipid complexes. Over 60 distinct O antigens are recognized and are designated by arabic numerals. Most species

possess several O antigens which they share with a number of other species (e.g., Escherichia, Shigella, Citrobacter and Proteus).

- **H antigens** – represent determinant groups on the flagella protein. Over 70 distinct H antigens are recognized. H antigens in many salmonella are disphasic. In phase I, the "specific phase" they are designated by the small letters of the alphabet, a to z, then as Z1, Z2, Z3 etc. phase II H antigens are designated by Arabic numerals (not implying any relationship with the similarly numbered O antigens).
  - **Vi Antigens**: are capsular (K) antigens, act as a protective factor for the O antigen by preventing phagocytosis and the bacterial action of serum. Some salmonellae (S. typhi) have these antigens.

3.2.1.1. Serologic Diagnosis

Widal test

The Widal test is a serologic technique which tests for the presence of salmonella antibodies in a patient's serum. When facilities for culture or antigen testing are not available, the widal test if performed reliably and interpreted with care (with clinical findings); can be of value in diagnosing typhoid and paratyphoid in endemic areas. It has no value in the investigation of salmonella food poisoning. There are slide and tube agglutination tests.

- When investigating typhoid, the patient's serum is tested for O and H antibodies (agglutinins) against the following antigen suspensions:

S. typhi O antigen suspension, 9, 12

S. typhi H antigen suspension, d

- When testing for paratyphoid, A, B, or C, the following antigen suspensions are required:
  - ⇒ S. paratyphi A, O antigen suspension, 1, 2, 12
  - ⇒ S. paratyphi A, H antigen suspension, a
  - ⇒ S. paratyphi B O antigen suspension, 1, 4, 5, 12
  - ⇒ S. paratyphi B, H antigen suspension, b, phase 1
  - ⇒ S. paratyphi C, O antigen suspension, 6, 7
  - ⇒ S. paratyphi C, H antigen suspension, c, phase 1

Interpretation of the Test Results

The strength of the reaction for the infecting serotype increases progressively to a maximum about the end of the third week and the demonstration of such a rising titer, e.g. four fold or greater rise, between tests made in the first and third weeks is highly significant. Serodiagnosis of typhoid fever is based on a four fold or greater rise between acute and convalescent antibody titers, or a single titer that is significantly higher than the mean baseline titer for the population. Positive results in a single test by no means prove the presence of enteric fever, nor negative results its absence.

Cause of raised O and H titers other than active typhoid include:

Previous salmonella infection, chronic salmonellosis associated with schistosomal infection, vaccination with typhoid vaccines, current infection with other salmonella species, chronic liver disease associated with raised globulin levels, and disorders such as rheumatoid arthritis, rheumatic fever, multiple myeloma, nephrotic syndrome, and ulcerative colitis.

3.2.2 Rickettsiaceae

The human pathogens in the family Rickettsiaceae are small bacteria of the genera rickettsia, orientia, and erlichia. They are obligate intracellular parasites and, except for Q fever, are transmitted to human by arthropods such as fleas, lice, mites and ticks. Many rickettsia are transmitted transovarially in the arthropod, which serves as both vector and reservoir. Rickettsial infections, except Q fever and the ehrlichioses, typically are manifested by fever, rashes, and vasculitis. They are grouped on the basis of their clinical features, epidemiologic aspects, and immunologic characteristics.

Rickettsia can be grown in the laboratory only in cultures of living cells. Diagnosis of most of the rickettsial diseases is facilitated by the development in the blood of infected patients of specific antibodies that can be detected by serologic tests.

3.2.2.1. Serologic Diagnosis

Weil Felix (WF) test

This test is dependent on cross-reaction that exists between the antigens of certain rickettsiae and those of selected strains of Proteus vulgaris and Proteus mirabilis.

Suspensions of three proteus strains, OX – 19, OX – 2 and OX- K, are added to dilutions of patient serum. After appropriate incubation, the tubes are examined for agglutination of the proteus suspension. The end point is determined.

Result interpretation:

Either a four – fold or greater rise in titer between acute and convalescent sera or a single specimen titer of greater than or equal to 1:320 is considered to be evidence of certain rickettsial infections.

Note: False negative reactions are common in scrub typhus.

False positive reactions may occur in Proteus infections, relapsing fever, brucellosis and other acute febrile illnesses.

Table 1.3. Weil Felix Reaction

Organisms	OX-19	OX-2	OX-K

Typhus group				
-	R. prowazeki	+++	+/-	-
-	R. typhi	+++	+/-	-
Scrub Typhus group				
-	R. trsutsugamushi	-	-	+++/-
spotted Fever group				
-	R. conori	+/++	+/++	-
-	R. conoripijperi	+/++	+/++	-
-	R. siberica	+/++	+/++	-
-	R. rickettsi	+/++	+/++	-

3.3. Serology of Streptolysin O (SLO) and Antistreptolysin O (ASO)

3.3.1. The Extra cellular Products of Streptococcus pyogenes

Streptolysin O (SLO) is a bacterial toxin produced by virtually all strains of *S. pyogens*. It is one of the two extracellular hemolysins (or cytolysins), the other being streptolysin S (SLS). Streptolysin O is released during infection as indicated by antibody production to it. The toxin is a protein with a molecular weight of approximately 70, 000 which, in its reduced state, brings about the lysis of red and white blood cells.

Properties of Streptolysin O

Streptolysin O is called so because of its oxygen liability, and it is quite distinct from SLS. It is hemolytically inactive in the oxidized form and is characteristics of a group of cytolytic toxins known as the oxygen-labile toxins, which are produced

by several different gram-positive bacteria and possess a number of common properties. They are activated by sulfhydrryl (SH) compounds, they appear to be antigenically related, and their biologic activity is completely inhibited by low concentrations (1.0µg/ml) of cholesterol and certain related sterols.

Hemolysis of erythrocytes occurs within minutes after the addition of SLO, and toxic effects of SLO have been demonstrated on several types of mammalian cells in culture. SLO is also cardiotoxic, inducing the release from atrial of acetylcholine, which poisons ventricles, probably causes its cardiotoxity. The first (the f site) contains two cystine residues and is responsible for the attachment of the molecule to the red blood cells, the second site(the t site) is concerned with the final hemolytic event.

It is evident that membrane cholesterol is the binding site of SLO, because only those cells that contain cholesterol in their membranes are susceptible to the toxin. In addition, SLO is inactivated only by the membrane lipid fraction that contains cholesterol. The addition of exogenous cholesterol to SLO inhibits toxic action, and treatment of erythrocyte membranes with alfalfa saponin or fllipin inhibits the absorption of SLO. These agents are known to bind to cholesterol in the membrane. However, the actual mechanisms that results in cell lyiss remains to be explained.

3.3.2. Antistreptolysin O (ASO)

The ASO antibody is a globulin, occurring mostly in the gamma globulin fraction of the serum. It can combine with and fix streptolysin O, neutralizing it in vitro and making it incapable of lysing red cells.

The serological test used for the detection of ASO relies on:

A. Antistreptolysin O can be specifically fixed with the antigen streptolysin O, inhibiting its hemolytic activity
B. The amount of ASO can be estimated by serial dilution of the patients serum in the presence of constant volumes of SLO to the point where there is still complete prevention of hemolysis, and
C. The presence of ASO in the serum is directly related to the production of SLO by the streptococcal bacteria in the infected patient.

Significance of the Antistreptolysin O Reaction

Streptolysin O is antigenic, eliciting the formation of antibodies that effectively neutralizes its hemolytic action. A high proportion of patients with streptococcal infections show an antibody response during convalescence; therefore, the measurement of serum antistreptolysin O (ASO) has become a valuable and reliable indicator of streptococcal infection,

particularly in cases of rheumatic fever and glomerulonephritis which are complications of streptococcal infections.

3.3.2.1. Tests for Antistreptolysin O

The most widely used test for SLO is the neutralization (ASO titration) test used to detect ASO in serum. This test is based on the fact that ASO can be specifically fixed to SLO in vitro, where it will neutralize its hemolytic activity. The test, therefore, by doubling dilution, estimates the amount of antibody that, in the presence of a constant dose of SLO, can completely inhibit hemolysis of a given number of red cells.

In the interpretation of ASO titers, many variables, including age, the severity of the infection, previous exposure to streptococcal infection, and the individual's ability to respond immunologically to the toxin, must be taken into account, since no set 'normal' titer has been established. Most healthy adults (99 percent) have ASO titers of 125 Todd units (or less). The original ASO test procedure was developed by Todd, whose name is still used to express the levels of antibody titer. One Todd unit is that amount of antibody that completely neutralizes two and one-half minimal hemolytic doses of SLO. Children, however, show fluctuating ASO titers from 5 to 125 Todd units. The usual titer normally decreases after 50 years of age, probably owing to a weeknd immunologic response.

A rise in ASO titer of at least 30 percent over the previous level is usually regarded as significant. In cases of rheumatic fever and glomerulonephritis, a marked increase in ASO titer is often seen during the symptom-free period preceding an attack of the illness. ASO titers in acute cases of rheumatic fever are usually between 300 and 1,500 Todd units and are usually maintained at high levels for a period of 6 months from the onset of disease. Drugs commonly used in the treatment of patients with rheumatic fever (e.g. sodium salicylate, aureum salts, and aminophenazone with phenlybutazone [Irgapyrin] do not affect the production of SLO in vivo, but antibiotics (e.g.penicillin, Aureomycin), hormones, and cortisone inhibit the production of the toxin.

Increased ASO titers have been found in a large number of diseases (e.g. scarlet fever, cholera minor, tuberculosis disease, pneumococcal pneumonia, and gonorrhea), although they are rarely above 500 Todd units, unless the patient has had a recent streptococcal infection. Very low titers are observed in all states of the nephritic syndromes, possibly as a result of a defect in formation, increased destruction of antibody protein, or loss of antibody protein in the urine.

A single high ASO titer is of little value to the clinician because a small number of healthy individuals have high titers.

Significance should only be attached to changes in ASO titers determined by serial titration.

In addition to the ASO titration, a particle agglutination test in which erythrocytes are coated with a crude mixture of streptococcal antigens is available. This test is good for screening, but has limited value as a quantitative test.

3.3.2.2. Antistreptolysin O Titration

Antistreptolysin O titration allows the quantitative analysis of the antibody, based on an internationally recognized unit system. The system defines a minimal hemolytic dose of SLO as that amount of toxin that will completely hemolyze 0.5 ml of a 5 per cent suspension of rabbit red blood cells, measured in Todd units.

Materials

1. Saline -0.85 per cent
2. Streptolysin O buffer

This is commercially available from a number of suppliers. It is prepared as follows:

7.4 gm sodium chloride

3.17 gm potassium phosphate

1.081 gm sodium phosphate

Add to 1,000 ml of distilled water. The final PH should be between 6.5 and 6.7.

The buffer may be stored at 4^0Cfor up to 1 week.

3. Streptolysin O

   This is available in dehydrated form from commercial supply houses and should be rehydrated just prior to use. Once rehydrated, the solution should not be subjected to vigorous shaking, and it must be used within 1 hour or discarded, because the active reagent is subject to inactivation by oxidation.

4. Red blood cells

   A 5 per cent suspension of fresh (not more than 1 week old) human red blood cells (group O) is most commonly used in this test, although rabbit red blood cells are equally sensitive to SLO. The cells must be washed three times in diluents, and the buffy coat (white blood cells) must be removed. The final centrifugation should be at 1, 500 rpm for 10 minutes, following which the packed red cells may be measured to achieve a 5 per cent suspension.

5. Test tubes,12x100 mm are commonly used (round bottom)

Procedure

1. Prepare dilutions of fresh or inactivated serum as follows, using SLO buffer as a diluents:

   1:10 -0.5 ml of serum 4.5ml of buffer

   1:100 -1.0ml of 1:10 serum dilution plus 9.0ml of buffer

1:500 -2.0 m of 1:100 serum dilution plus 8.0ml of buffer

The first two serum dilutions are usually sufficient for preliminary titration

2. Set up the test according to the protocol given in this table.

TABLE 1. 4. Protocol for Antistreptolysin O Titration														
Serum Dilutions Red cell	1:10					1:100								1:500
	SLO													
Control	Control													
Tube		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Add serum dilution, ml		0.8	0.2	1.0	0.8	0.6	0.4	0.3	1.0	0.8	0.6	0.4	0.2	0	0
Add buffer solution, ml		0.2	0.8	0	0.2	0.4	0.6	0.7	0	0.2	0.4	0.6	0.8	1.5	1.0
Shake gently to mix															
Add streptolysin O ml		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5
Shake gently to mix, incubate at 37^0 for 15 minutes															
Add 5 percent red cell Suspension, ml		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Shake gently to mix, incubate at 370 for 45 minutes, shaking tubes after first 15 minutes, following incubation centrifuge tubes for 1 minute at 1, 500 rpm															
Todd unit value		12	50	100	125	166	250	333	500	625	833	1250	500	---	----

Interpretation

The ASO titer expressed in Todd units is the reciprocal of the serum dilution that completely neutralizes the SLO. For example, a serum showing no hemolysis in tube 1 through 4, a trace of hemolysis in tube 5 and marked to complete hemolysis in the remaining tubes is reported as containing 125 Todd units.

Before reporting results, always ensure that the controls give the expected results.

Rapid latex Agglutination Antistreptolysin O Procedure

The rapid latex agglutination antistreptolysin O (ASO) procedure is based on the principle that, if polystyrene latex particles are coated with streptolysin O antigen, visible agglutination will be exhibited in the presence of the corresponding antistreptolysin O antibody.

Materials

1 ASO latex reagent coated with streptolysin O. Store at 2 to 8^0C. Mix well before use.

2 0.9 per cent NaCl solution. This is a saline solution containing sodium azide as a preservative.

3 Positive control serum. A prediluted serum containing at least 200 U/ ml of ASO. This control should exhibit visible agglutination at the end of the 3 minute test period.

4 Negative control serum, a preidluted serum containing less than 100 U/ ml of ASO This control should exhibit a smooth or slightly granular appearance at the end of the 3- minute test period.

5 Glass slides with 6 wells. Use only the glass slide provided. The slide should be rinsed in distilled water and thoroughly dried with a soft cloth or tissue after each use.

Additional materials required, but not provided in the kit:

1. Applicator sticks
2. Timer
3. 12x75 mm test tubes
4. Pasteur pipettes and rubber bulb
5. Serologic pipettes and safety bulb
6. 50 µl disposable pipettes and safety bulb
7. High- intensity direct light

Procedure (Screening Test)

Note: All reagent and specimens should be at room temperature before testing.

1. Label a 12x 75mm test tube for each patient to be tested.
2. Pipette 1ml of saline into each test tube.
3. Add 1 drop of patient serum to each of the appropriately labeled test tubes using a Pasteur

pipette. Cover the tube and mix the dilution thoroughly by inverting the tube several times.

4. Label 1 division of the 6 cell slide for the positive control, negative control and the respective patient sera to be tested.
5. Pipette 50µl of the controls and patient sera onto the appropriately labeled cells. Use a fresh pipette for each specimen.
6. Add 1 drop of latex reagent to each cell.
7. Mix each specimen with a separate applicator stick. Spread the mixture evenly over the cell.
8. Rotate the slide for exactly 3 minutes.
9. Examine immediately with a bright source of direct light.

Interpretation

Agglutination indicates a positive result and no agglutination indicates a negative result, provided that the controls have given the expected results.

Agglutination demonstrates 200 U/ ml or more of ASO. Positive result should be retested quantitatively. In semi quantitative testing, the U/ml of the highest dilution of serum to produce visible agglutination is the reported value.

Quality control

Run controls parallel with test samples.

3.4. Toxoplasmosis

It is caused by the protozoan *Toxoplasma gondii,* a member of the sprozoa.

The tachyzoite directly destroys cells and has a predilection for parenchymal cells and those of the reticuloendotelial system.

Humans are relatively resistant, but a low grade lymph node infection resembling infectious mononucleosis may occur. When a tissue cyst ruptures releasing numerous bradyzoites, a local hypersensitivity reaction may cause inflammation, blockage of blood vessels and cell death near the damaged cyst.

The organism in humans produces either congenital or postnatal toxoplasmosis. Congenital infection develops only when non immune mothers are infected during pregnancy. Postnatal toxoplasmosis is usually much less severe.

Congenital infection leads to still births, intracerebral calcification and psychomotor disturbance when the mother is infected for the first time during pregnancy. Prenatal toxoplasmosis is a major cause of blindness and other congenital defects.

Most human infections are asymptomatic. However, fulminating fatal infections may develop in patients with AIDS presumably by alteration of a chronic infection to an acute one or changing hosts resistance.

Some acquired immunity may develop in the course of infection. Antibody titers in mothers as detected in either blood or milk tend to fall within a few months. Yet, the fact that prenatal infection is limited to infants born of mothers who were first exposed during their pregnancy, strongly suggests that the presence of circulating antibody is at least partially protective.

3.4.1. Serological tests

The Sabin Feldman dye test depends upon the appearance in 2-3 weeks of antibodies that will render the membrane of laboratory cultured living *T. gondii* impermeable to alkaline methylene blue. Thus, organisms are unstained in the presence of positive serum. This test is being replaced by the IHA, Indirect FAT, and ELISA tests. A CFT may be positive (1:8 titer) as early as 1 month after infection, but it is valueless in many chronic infections. The Indirect FAT and IHA tests are routinely used for diagnostic purposes. Blood (Buffy coat of heparinzed sample), bone marrow, CSF and other body fluids can be tested. In addition, Frenkel's intracutaneous test is use full for epidemiological surveys.

3.5 Hydated Disease

Hydated disease is caused by the accidental ingestion of eggs of the tape worm *Echinococcus granulose* in food or water or from hands contaminated with dog faeces. Humans are not the natural intermediate hosts of *E. granulosus*. The normal intermediate hosts are sheep, cattle and goats, with dogs becoming infected by eating tissue containing hydrated cysts from these animals. Infections can produce serious symptoms depending on the site and size of the hydatid cyst and host response.

Serological diagnosis of hydatid disease

In general, the sensitivity of serological tests is affected by the site and condition of a hydatid cyst. Sensitivity is higher with liver cysts than with lung cysts. Dead or calcified cysts may give negative results. False negative results may be obtained from patients with circulating immune complexes. For most of the tests that have been developed, reagents are not generally available. But testing serum for antibodies produced in response to infection and where available testing for cystic fluid antigens is diagnostic.

Review Question

1. Discuss the different stages of syphilis
2. Explain the difference between treponemal and non-Treponemal tests for syphilis
3. List the stages of syphilis and possible laboratory diagnostic tests for each stage
4. Explain immunologic manifestations in syphilis

5. List non treponemal serological tests for syphilis and discuss their principles
6. List treponemal tests and state their applications
7. State the use, principle and draw backs of widal test
8. State the interpretation of the widal test results
9. Describe the use, principle and draw backs of weil Felix test
10. State the interpretation of weil Felix test results
11. Explain toxoplasmosis.
12. Describe different laboratory tests for toxoplasmosis
13. Describe serological tests for hydrated diseases.

CHAPTER FOUR
COMMON SEROLOGIC TESTS FOR VIRAL INFECTIONS

Learning Objectives

At the end of this chapter the students should be able to:

1. Describe and /or perform serologic tests of viral infections (HIV/AIDS, Hepatitis,

Infectious mononucleosis, Rubella infection, and cytomegalovirus),

2. Identify factors affecting each serologic test and minimize this effect
3. Explain how to read, interpret and report the results of each serologic test.

Introduction

4.1. Serologic tests for HIV/AIDS

Several laboratory methods are available to screen blood, diagnose infection, and monitor disease progression in individuals infected by HIV. These tests can be classified into those that: 1) detect antibody, 2) identify antigen, 3) detect or monitor viral nucleic acids, and 4) provide an estimate of T-lymphocyte numbers (cell phenotyping).

The isolation of HIV, its nucleic acid and methods used to detect HIV antigen are mainly used to detect early HIV infection before antibodies develop. They are also used to detect the progression from asymptomatic to symptomatic AIDS infection by monitoring an increase in p24 antigen. In patients who have developed the signs and symptoms of AIDS, assessment of T-lymphocytes and viral load concentration are important along with the diagnosis and

treatment of opportunistic infections. It is also possible to detect HIV-2 antibodies, proteins, and genes through the modification of test methods for HIV-1.

4.1.1. HIV Antibody Tests

Serologic diagnosis of HIV infecton is based on a multi-test algorithm for detecting antibodies to HIV by using screening and confirmatory tests. Screening tests provide presumptive identification of specimens that contain antibody to HIV. An HIV test kit is a good screening test when it can rule out all the people who do not have HIV. Tests like EIAs or simple/rapid imunodiagnostics are selected for their high sensitivity of detecting antibodies to HIV for screening.

Suplemental or confirmatory tests, such as western blot (WB), can be used to confirm infection in samples that are initially reactive on conventional EIAs.

- An HIV test kit is a good confirmation test when it can verify all cases who are truly HIV positive.
- Alternatively, repetitive testing incorporating EIAs or rapid tests selected for their specificity may be used to confirm wether specimens found to be reactive for HIV antibodies with a particular screening test are specific to HIV.For practical purposes, resource-poor settings depend heavily on EIA and rapid tests for screening and confirmation.

4.1.1.1. HIV Antibody Test Algorithm

A test algorithm for seroloic diagnosis of HIV-infection is the sequence in which assays are performed to detect HIV antibody in a body fluid.

The most common referenced test algorithm employs an EIA to screen specimens and then those found to be positive are confirmed by western blot testing. This is so called conventional algorithm and it has several limitations:

- western blot is expensive and requires techical expertise.
- western blot often yields indeterminate results with certain types of specimens
- Both ELISA and WB are time consuming and require a well equipped laboratory infrastructure.

Several alternative testing algorithms exist for the serolgic diagnosis of HIV infection that are based on a combination of screening assays, without using WB. They can be grouped into parallel and serial testing algorithm.

A. Parallel testing algorithm

Sera are simultaneously tested by two assays. True positive sera are concordantly reactive by two different intial assays. A true-netgative sipecimen in the algorthm is defined as being concordantly negative in the two initial

assays. Sera yielding discordant results between the two assays are tested in a third assay, and the outcome of the later assays is considered definetive.

B. Serial testing algorithm

The serial testing algorithm is most consistent with the proposed testing strategies of WHO/UNAIDS. In the serial algorithm, all speimens are tested by a first test that is highly sensitive. Specimens are considered as true negative if they react negatively in the first test. Specimens reactive in this assay are retested by a second assay that has a high specificity (this second assay must be one which possesses dissimilar antigen presentations than that of the first assay). If specimens are concordantly positive by the two assays, they are considered as true-positives. Discordantly reactive sera are further tested by a third assay, whose outcome is considered as definitive.

4.1.1.2. Common HIV Antibody Tests

A. Enzyme Linked Immunosorbent Assays (ELISA)

Enzyme Linked Immunosorbent Assays relies on a primary antigen-antibody interaction.

Since 1985 EIA s have progressed considerably from first to fourth generation assays:

First generation assays were based on purified HIV whole viral lysates, have poor sensitivity and specificity.

Second generation assays used HIV recombinant proteins/ or synthetic peptides which enabled the production of assays capable of detecting HIV-1 and HIV-2. It has had improved specificity, but has similar overall sensitivity to that of first generation assay.

Third-generation assays used the solid phase coated with recombinant antigens and/or peptides and similar recombinant antigens and peptides conjugated to a detection on enzyme or hapten that could detect HIV-specific antibodies bound to a solid phase.These assays could detect IgM early antibodies to HIV, in addition to IgG, thus resulting in a reduction of the sero-conversion window (2-4 week time period).

Fourth generation assays are very similar to third-generations test, but have the ability to detect simultaneously HIV antibodies and antigens. Hence, reduce window period.

Characteristics of Enzyme Linked Immunosorbent Assays

Enzyme Linked Immunosorbent Assays are best performed at a regional or national laboratory since they require well-trained and skilled laboratory technicians, technologically advanced equipment (incubators, Washers and spectrophotometers) that requires maintenance and a constant source of electricity. Enzyme Linked Immunosorbent Assays is most efficient for laboratories that process a large number of specimens (100 or more) daily or for batch testing which is common in HIV sentinel surveillance. Because of test design, they are not

suitable or cost-effective to run on a small number of specimens. However, if a laboratory processes at least 50 specimens each day on a regular basis, ELISA may still be more appropriate than rapid tests. Enzyme Linked Immunosorbent Assays may have limited application in rural settings where the laboratory infrastructure and equipment may be insufficient.

Performing an ELISA

Enzyme Linked Immunosorbent Assays can be performed with serum, plasma, urine, oral fluids, or dried blood spots (once eluted). They can take from 2 to 4 hours to perform (including specimen preparation and dilution) and an additional 3 to 4 hours if a screening result has to be confirmed. Manufacturer's instructions provided with the specific ELISA used should be followed.

General steps for performing an ELISA

1. Dilute the specimen in the specimen buffer and put it in a micro well plate containing HIV antigen already bound to the plate.
2. Incubate the plate as per protocol and then wash as indicated.

3. Add antihuman immunoglobulin-enzyme conjugate, which will react with the HIV specific antibody if present
4. Incubate
5. Wash the plate, add the substrate and incubate as prescribed
6. Add a stopping solution to terminate the enzyme reaction and read the absorbance of the solution using spectrophotometer.

A positive reaction has occurred if the specimen in the specimen well changes color or becomes colored, which indicates the presence of HIV-specific antibody in the specimen. The reaction is best read quantitatively with an ELISA plate using spectrophotometer (ELISA reader).

The following are critical to the success of conducting an ELISA:

- Use of test kits that are not expired
- Calibrated and well-maintained equipment
- Adherence to dilution and incubation times described in the manufacturer's instructions.
- Use of deionized water
- Use of a spectrophotometer to read results accurately and objectively
- Training with the technology being used

- Consistent source of power without outages that would affect the storage of reagents or the functioning of equipment

Quality control

Run controls with the patient sample as per the manufacturers' instructions.

Fourth generation Enzyme immunoassay sorbent (ELISA) for HIV

Serological evidence of HIV infection may be obtained by testing for HIV antigens or antibodies in serum or plasma of individuals suspected of HIV infection. Antigens can generally only be detected during the acute phase and during the symptomatic phase of AIDS. Antibodies to HIV-1and /or HIV-2 can be detected throughout virtually the entire infection period, starting at or shortly after the acute phase and lasting till the end stage of AIDS. The use of highly sensitive antibody assays is therefore an established approach in sero diagnosis of HIV infection and in the screening of blood and blood products.

Progressive improvements in assay sensitivity have reduced the so called window phase, i.e. the time between infection with the HIV virus and the moment that antibodies to HIV can

be detected by sensitive HIV antibody tests. Further shortening of this widow period can be achieved by the incorporation of HIV antigen detection in such HIV antibody tests enabling the detection of infected individuals of the earliest possible moment.

For example, *vironostika HIV uni- form II Ag/Ab* is a fourth generation ELISA: based on a one step sandwich principle. A mixture of HIV antigens and HIV antibodies coupled to horse reddish peroxides (HRP) serves as the conjugate with tetramethyl benzidine (TMB) and peroxide as the substrate. Upon completion of the assay, the development of color indicates the presence of HIV antibody or HIV antigen, while no or low color development suggests the absence of HIV antibodies or antigens.

Procedure

1. Fill the strip holder with the required number of microelisa strips – remove the strip sealers.
2. Pipette 100µl specimen diluents in to all wells, i.e including control wells
3. Pipette 50µl sample or control into assigned wells. Include three negative controls and one anti-HIV -1 positive control in each stripe holder. If desired, one anti –HIV-2 positive control .Always pipette the controls after pipetting the samples.

4. Mix well (e.g, using a microshakar) for 15 second
5. Wash and soak each well six times with phosphate buffer.
   - Incomplete washing will adversely affect the assay outcome
   - Aspirate the well contents completely into a waste flask.

   Then fill the wells completely with phosphate buffer avoiding overflow from one well to another and allow to soak for 30 to 60 seconds. Aspirate completely and repeat the wash and soak procedure five times for a total of six washes.
6. Pipette 100µl TMB substrate into each well. Do not mix or shake
7. Incubate the strips at 15 to 30^0C for 30 ± 2 minutes.
8. Stop the reaction by adding 100µL 1mo /L sulfuric acid to each well, use the same pipetting sequence and time intervals used for TMB substrate addition .

   - Plate should be read within 15 minutes
9. Blank the reader on air, i.e. without strip holder and strips, and read the absorbance of the solution in each well at 450 nm (single wavelength) or 450nm and 620 to 700nm as reference (dual wavelength) .

Results

Manual calculations

Calculations must be made separately for each strip holder

NC = absorbance of negative control

P_{C1} = Absorbance of the anti-HIV-1 positive control

P_{C2} = Absorbance of the anti –HIV -2 positives control

P_{C3} = Absorbance of the HIV -1 antigen positive control

Qualification of NC values

1. Nc must be < 0.250 → eliminate any NC ≥ 0.250
2. Determine the mean (N_{CX}) value of the remaining controls
3. Nc must be ≤ 1.4 Ncx → eliminate any Nc > 1.4 Ncx and recalculate Ncx
4. Nc must be ≥ 0.6Ncx → 1eliminate any Nc < 0.6 Ncx and recalculate Ncx
5. Repeat step 3 and 4 until no more outlier are found

Assay validity

An assay run is valid if,

1. More than half the negative control remain;
2. P_{C1}- Ncx ≥ 0.6000
2. P_{C2}- Ncx ≥ 0.6000(if used)
3. P_{C3} ≥ 0.400(if used)

Cut –off value

If the test run is valid, calculate the cut-off value Ncx +0.100

A test sample is reactive if sample absorbance is ≥ cut-off value

A test sample is non reactive if sample absorbance is < cut – off value

Calculations Example

Nc = 0.089, 0.,096 , 0.088

Ncx = 0.091

P_{c1} = 1.549

P_{c2} = 1.523

P_{c3} = 1.398

Eliminate any aberrant control

Nc ≥ 0.250→ none eliminated

Nc > 1.4 Ncx → 1.4 (0.091) = 0.127 →none eliminated

Nc < 0.6 NCx → 0.6 (0.091) = 0.055 → none eliminated

Calculate cut off value

Cuff off = Ncx + 0.100

=0.091 + 0.100 = <u>0.191</u>

HIV Antigen Testing

<u>P_{24} antigen</u>

Enzyme immunoassay for HIV-1 antigen detects primarily uncompleted P_{24} antigen. This procedure is applicable to

blood or CSF testing as evidence of an active infection and can be diagnostic before sero conversion, can predict a patient's prognosis, and is useful for monitoring response to therapy. Disadvantage of the procedure includes poor sensitivity, the inability to detect in patients with a high titer of P_{24} antibody, and the failure of the method to detect HIV-2 antigen. Antibodies to P_{24} antigen are a better predictive marker progression than P_{24} antigen

B. Rapid Tests

General Description

Interests in the development of HIV antibody tests that provide same-day results and that do not require additional reagents or equipment not contained in the kit led to the currently available HIV rapid tests. Rapid tests are based on four immunologic principles: particle agglutination, immunodot (dipstick), immunofiltration (flow through device), and immunochromatography (lateral flow).

Most HIV rapid tests contain antigens to HIV -1 and HIV-2 and detect antibodies to both. A positive test result is indicated by clumping, a spot dot or line depending on the test format. The sensitivity and specificity of the latest generation of rapid tests are similar to those of ELISA. Many rapid tests are under evaluation or are currently in use in developing countries for screening, diagnostic and surveillance purposes.

Characteristics of Rapid Tests

Rapid tests are useful for small laboratories that routinely perform fewer than 100 HIV tests per day, for laboratories without electricity or equipment, and for geographic areas with limited laboratory infrastructure. In some instances, even if a laboratory performs more than 100 tests per day, but only during a limited time in a year, rapid tests may be more appropriate than ELISA. A result can usually be obtained in less than 45 minutes, and it is easy to interpret. However, some training is required to correctly perform the test and interpret the results. The test kits generally contain all reagents needed to run the assay, no additional reagents or equipment is required. Many rapid tests do not require electricity, special equipment, refrigeration, or highly skilled staff although a few require refrigeration for heat- sensitive reagents.

Recommendation

Rapid tests are useful in settings where ELISA are not feasible or practical and in geographic areas with limited laboratory infrastructure. Rapid tests may be appropriate for hard-to-reach populations (e.g. Injection drug users, female sex workers) or geographically remote populations for whom HIV test results may need to be provided on site on the same day of specimen collection.

Interpretation of HIV Antibody Rapid Test Results

1. All serum/plasma is first tested with one rapid assay (T1) which is highly sensitive.
2. Serum that is non-reactive on the first test is considered HIV antibody negative.
3. Any serum found reactive on the first assay (T1) is retested with a second highly specific rapid assay (T2) based on a different antigen and/ or different test principle.
4. Serum that is reactive on both tests is considered HIV antibody positive

5. Any serum that is reactive on the first test but non-reactive on the second test should be retested with the first test kit (T1). If the result is non- reactive, then it should be considered HIV antibody negative. The reactive result at the first (T1) was probably a technical error when conducting (T1).
6. When serum that is reactive on the first test, (T1) and negative on the second test (T2) is again reactive with first test (T1), then one should repeat the second test (T2) in order to rule out a technical error. If the result is positive with (T2), then it is considered HIV antibody positive. It means that there was a technical error associated to (T2).

   If however, the test result is negative with T2, then a tie- breaker (T3) is needed. If the result of the tie-breaker test is positive the sample is reported as HIV antibody positive or if the result is negative the sample is considered negative.

These tie breaker test kits will be needed in about 2% of cases. Therefore, it is suggested that, for economic reason, tiebreakers should be kept only at referral hospitals.

Quality control

Follow the manufacturer's instructions for quality control.

C. Western Blot

Western blot is a technique in which proteins are separated electrophoretically, transferred to membranes, and identified through the use of labeled antibodies specific for protein of interest.

In the western blot (WB) assay, HIV virus is disrupted and HIV proteins are separated by molecular weight into discrete bands by electrophoresis on polyacrylamide gels. The viral proteins are then transferred onto nitrocellulose sheets and cut into strips. Individual strips are incubated overnight with patient serum, washed, and incubated with anti-human immunoglobulin conjugated with enzymes or biotin. After the addition of the appropriate substrate, color develops to show discrete bands where antigen - antibody reactions have occurred.

Interpretation of the results of the tests is generally governed by Centers for Disease control guidelines and individual laboratory experiences. The Centers for Disease Control recommends that tests be considered positive when the gp41 band appears alone or when an envelope antibody (gp41, gp120, or gp160) appears in combination with another HIV characteristic band (p15, p18, p24, gp41, p51, p55/ p66, gp120, or gp160). In some cases, kits supplied by commercial

companies will have special interpretive instructions, and these should obviously be followed when using the particular kit. The test is considered negative when no bands appear. If there is isolated reactivity to a single HIV protein or a pattern of reactivity to multiple proteins from the same viral gene product (i.e. polymerase gene products: p31, p51/66 only), the test result is considered doubtful (or indeterminate). In this case, a follow-up specimen from the patient should be collected in 6 months and test repeated in case the patient is in the early stages of HIV infection.

False- positive WB reactions may occur in healthy individuals, infections in bilirubinemia, in connective tissue diseases, and in-patients with human leukocyte antigen (HLA) antibodies.

Western blot is the most widely used supplementary test for confirming HIV ELISA antibody tests. The test has certain disadvantages,-it is cumbersome to perform, it requires overnight incubation and interpretation is often difficult.

4.1.1.2.1. Explanation of the Meaning of HIV Antibody Test Result

A negative test result means that the person is either not infected or is so recently infected, that the test could not detect the infection. In the later case, the person could be in the **'window period'**. During this period, which may last 3 to 6

months after the initial infection, the person could be infected with HIV and can infect others, but will have a negative test and possibly with no physical complaints. A positive test result means that the person is infected with HIV and can transmit it to others.

HIV test algorithm in Ethiopia

After assessing the current situation in the country regarding the technical and logistic problems of ELISA machines and kits, the use of two rapid tests of different principles is recommended as the minimum HIV test algorithm and a third rapid test as **a tie breaker** in cases where there is **discordance** between the first and second test. This strategy shall be followed at all levels of health care delivery system (hospitals, health centers, clinics etc.) in government, private and NGO settings. However, health facilities having ELISA machine may continue to use ELISA test kits.

4.2. Serology of Hepatitis Viruses

4.2.1. Introduction

Viral hepatitis is the most common liver disease in the world. Although the target organ for each of these viruses is the liver, they differ greatly in their structure, mode of replication mode of transmission and in the course of the diseases they cause.

There are six types of hepatitis viruses: Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV),

Hepatitis D virus (HDV), Hepatitis E virus (HEV) and Hepatitis G virus (HGV).

Each of the hepatitis viruses infects and damages the liver causing the classic Icteric symptoms of jaundice and the releases of liver enzymes. The specific virus causing the disease can be distinguished by the course, nature, and serology of the disease.

4.2.2. Hepatitis A virus (HAV)

Which was formerly known as infectious hepatitis is caused by a picornavirus and spread by the feco-oral route. It has an incubation period of approximately 1 month after which icteric symptoms start abruptly and does not cause chronic liver disease, but rarely causes fatal disease. Hepatitis A virus (HAV), is ingested and probably enters the blood stream through the oropharynx or the epithelial lining of the intestines to reach its target, the parenchymal cell of the liver. It can be localized by immunofluorescence in hepatocytes and kupffer's cells. Virus is shed in large quantity in to the stool approximately 10 days before symptoms of jaundice appear or antibody can be detected. Icterus resulting from damage to the liver occur when antibody is detected and cell-mediated immune responses to the virus occur. Antibody protection against reinfection is lifelong. The finding of IgM anti-HAV in a patient with acute viral hepatitis is highly diagnostic of acute

HAV. Demonstration of IgG anti- HAV indicates previous infection.

4.2.2.1. Laboratory Diagnosis

The diagnosis of HAV infection is generally made on the basis of the time course of the clinical symptoms, the identification of a known infected source, and most reliably the results yielded by specific serologic tests. The best way to demonstrate an acute HAV infection is by finding anti-HAV IgM, as measured by an ELISA or RIA. Virus isolation is not routinely performed since efficient tissue culture systems for growing the virus are not available.

4.2.3. Hepatitis B virus (HBV)

This was previously known as serum hepatitis. It is a member of hepadnavirus with a DNA genome and can be spread parenterally by blood or needles, by sexual contact and perinataly. It has a median incubation period of approximately 3 months after which icteric symptoms start insidiously. HBV can cause acute or chronic symptomatic or asymptomatic disease. Detection of both the hepatitis B surface antigen (HBs Ag) and the hepatitis B e antigen (HBe Ag) components of the virion in the blood indicates the existence of an ongoing active infection. Hepatitis B surface antigen particles continue to be released in to the blood even after virion release has ended and until infection is resolved. The major source of

infectious virus is blood, but HBV can be found in semen, saliva, vaginal and menstrual secretion and amniotic fluid. The most efficient way to acquire HBV is through injection of the virus into the blood stream, common, but less efficient routes of infection are sexual contact and birth. Antibody (as generated by vaccination) can protect against infection. However, the large amount of HBs Ag in serum binds to and blocks the action of neutralizing antibody which limits the antibody's capacity to resolve an infection.

Laboratory Diagnosis

The initial diagnosis of hepatitis can be made on the basis of the clinical symptoms and the presence of liver enzymes in the blood. However, the serology of HBV infection describes the course and nature of the disease. Acute and chronic HBV infections can be distinguished by the presence of HBsAg and HBeAg in the serum and the pattern of antibodies to the individual HBV antigens.

The detection of hepatitis B core antigen (HBcAg) is the best correlate to the presence of infections virus. A chronic infection can be distinguished by the continued finding of HBeAg, HBsAg or both and a lack of detectable antibody to these antigens. During the symptomatic phase of infection, detection of antibodies to HBeAg and HBsAg is obscured since the antibody is complexed with Antigen in the serum. The best

way to diagnose a recent acute infection, especially during the period when neither HBsAg nor anti-HBsAg can be detected (the window) is to measure IgM anti-HBcAg. Serum that is collected in the acute stage of illness can be tested by ELISA, counter immunoelectrophoresis and Reverse Passive Hemagglutination test (the most commonly employed test because it is the least expensive).

4.2.4. Hepatitis C virus (HCV)

Also called non A non B virus. It is a flavivirus with an RNA genome, spread by the same routes as HBV, but usually causes chronic liver disease. The chronic hepatitis often leads to cirrhosis and potentially to hepatocellular carcinoma. Antibody to HCV is not protective and findings yielded by experimental infection of chimpanzees indicate that immunity to HCV may not be life long.

Laboratory Diagnosis

The diagnosis and detection of HCV infections are based on recognition of anti-HCV antibodies. Seroconversion occurs within 7 to 31 weeks of infection. However, antibody is not always present in viremic people. ELISA is used for screening the blood supply from normal donors, but may not be sufficient for immunocompromised patients and those receiving hemodialysis. Reverse transcriptase PCR branched chain DNA and other molecular techniques can detect HCV RNA in

seronegative people and have become key tools in the diagnosis of HCV infection.

4.2.5. Hepatitis G virus (HGV)

HGV resembles HCV in many ways. HGV is a flavivirus, is transmitted in blood and has a tendency for chronic hepatitis disease. HGV is identified by detection of the genome by reverse transcriptase PCR or other RNA detection methods.

4.2.6. Hepatitis D virus (HDV)

It is also called delta virus that requires the presence of HBV for its complete life cycle. It occurs only in patients who have active HBV infection. HBV provides an envelope for HDV RNA and its antigen. Delta agent exacerbates the symptoms caused by HBV. Like HBV, the delta agent is spread in blood, semen and vaginal secretions. Although antibodies are elicited against the delta agent, protection probably stems from the immune response to HBs Ag.

Laboratory Diagnosis

The only way to determine the presence of the agent is by detecting the delta antigen or antibody; ELISA and RIA procedures are available. The delta antigen can be detected in the blood during the acute phase the disease in a detergent treated serum sample.

4.2.7. Hepatitis E virus (HEV)

The E stands for enteric or epidemic. It is predominantly spread by the feco-oral route especially in contaminated water. The symptoms and course of HEV disease are similar to those of HAV disease. It causes only acute disease. However, the symptoms for HEV may occur later than those of HAV disease. Specific test for IgM and IgG anti hepatitis E virus antibodies are diagnostic of HEV.

4.3. Serology of Infectious mononucleosis (IMN)

Infectious mononucleosis is also called a glandular fever, an acute infectious disease that primarily affects the lymphoid tissue, caused by Epstein Barr Virus (EBV). The virus enters the body via the respiratory tract and replicates with in the epithelial cells of the nasopharynx and salivary glands. Lysis of cells of the salivary glands releases EBV into saliva. Exchange of saliva is important in the transmission of EBV leading to IMN. During the course of the disease, B lymphocytes become infected and a state of latency is established in which the viral genome persists within the B cells. Cytotoxic T lymphocytes recognize and the virally infected B cell & epithelial cells. The T lymphocytes develop cellular abnormalities that are seen as atypical lymphocytes that characterize IMN (despite the name of this disease, the abnormal cells are lymphocytes and not monocytes). The symptoms and signs of IMN include a sore throat, low grade

fever, enlarged and tender lymph nodes. The virus has been associated with subsequent development of two forms of cancer; Burkett's lymphoma and naso pharyngeal carcinoma in different population group.

Three distinct groups of antibodies are found in infections mononucleosis:-

- Heterophil antibodies
- Epstein Barr virus (EBV) antibodies
- Hetero antibodies

Heterophil antibodies

Are antibodies that react with an antigen entirely different from and phylogenetically unrelated to the antigen responsible for their production. Are agglutinins that react particularly to sheep and horse red cells and are mainly class IgG. Are detected by Paul – Bunnell test. Antibodies to EBV are produced early in the disease and can be detected by complement fixation tests and Immunofluorescence techniques. Heterophil antibodies are present in low titer in the serum of normal persons and are known as forssman antibodies. They resemble the antibodies found in IMN in that they agglutinate sheep red blood cell, but differ from them in that they are absorbed by an emulsion of guinea pig kidney which is rich in forssman antigen and are not absorbed by beef cell which are poor in forssman antigen.

In cases of serum sickness or sensitization to animal (usually horse) serum, another type of sheep red cell agglutinating antibody is found and may be present in high titer. However, this is again distinguished from the antibody of IMN by being absorbed by guinea pig kidney and froms forssman antibody by being absorbed by beef red cells. This comparison is used as the basic for presumptive and differential tests. The sheep cell agglutinins of IMN can be distinguished from those of serum sickness and other conditions by means of a differential test using absorption with guinea pig kidney and beef red cell Antigens. The antibody that can be removed by absorption with guinea pig kidney is known as the forssman antibody and the guinea pig kidney as the forssman antigen.

- The classical sheep red cell agglutination test is carried out in two steps.
  1. The presumptive test of Paul - Bunnell
  2. The differential test of Paul Bunnell and Davidsohn
- Modifications of these classical procedures utilize horse red cells instead of sheep red cells.
- Under normal circumstances, rapid screening tests for IMN are done for the presence of heterophil antibody. Horse red cells are usually used rather than sheep red cells, as they are more sensitive to heterophil antibodies.

- Persons suffering from IMN begin developing heterophil antibody shortly after the appearance of the symptoms, usually during the first 2 weeks.
- Highest titers are found during the second and third weeks of the illness. The titer, however, bears no relationship with the severity of the illness.

Note Heterophil sheep cell agglutinins appear in only 50 -80% of cases of IMN

- Negative result can be obtained when the disease is present. Negative tests therefore, don not rule out the possibility of the disease.
- The test for heterophil antibodies is of confirmatory diagnostic importance in case of IMN with typical clinical and hematologic findings.

Recently, faster and easier screening tests have been introduced and have replaced the laborious presumptive and differential tests.

- These tests are done on a slide. The serum from the patient is mixed thoroughly with guinea pig kidney on one spot of the slide and with beef red cell stromata on another spot.
- The unwashed preserved horse red cells are added immediately to both spots. Agglutination is observed on both spots of the slide one minute after the final mixing.

Interpretation of slide agglutination

If agglutination is stronger on the spot where the guinea pig kidney suspension was mixed with the patient's serum, the test is positive. If it is stronger on the spot where the beef red cells were mixed with the patients serum, the test is considered negative. If agglutination is equal on both spots, the test is negative. If no agglutination appears on either spots, the tests is negative.

N.B

- The glass slide used for these rapid screening tests must be carefully cleaned under running water. Use of detergent could cause errors in the result. Most of the widely used immunologic assays for IMN are highly sensitive.

4.4 Rubella Infection

Introduction

Acquired rubella, also known as German or 30 days measles, is caused by an enveloped, single stranded RNA virus of the togovaridae family. Because the virus is endemic to humans, the disease is highly contagious and transmitted through respiratory secretions.

Contracting the infection or vaccination against rubella are the only routes to develop immunity. In patients suffering from a primary rubella infection, the appearance of both IgG and IgM antibodies is associated with the appearance of clinical sings and symptoms when present.

IgM antibodies become detectable a few days after the onset of sign and symptoms and reach peak level at 7 to 10 days. The presence of IgM antibody in a single specimen suggests that the patient has recently experienced a rubella infection. Demonstration of an equivalent increase in IgG antibody concentration between the acute and convalescent specimens is suggestive of either a recent primary infection or anamnestic antibody response to rubella in an immune individual. If both IgM and IgG test results are negative, the patient has never suffered from rubella infection or been vaccinated. If no IgM is demonstrable, but IgG is present in paired specimens, the patient is immune.

Testing for IgM antibody is invaluable in the diagnosis of congenital rubella syndrome in the neonate. IgM does not cross an intact placental barrier, therefore, demonstration of IgM in a single neonatal specimen is diagnostic of congenital rubella syndrome.

Diagnostic Evaluation

Historically, HAI antibody testing has been the most frequently used method of screening for the presence of rubella antibodies. Despite wide acceptance and use of these other assays, HAI testing continues to be the reference method for detection and quantification of rubella antibody.

Latex procedures provide more rapid and convenient alternative to HAI. If more quantitative results are desired, EIA and Fluorescent immunoassay (FIA) appear to be as reliable as HAI.

- ⇨ Wide spread use of EIA for assessment of immune status (IgG) and recent infection (IgM) should soon result in simplification of rubella serology. EIA can be used to measure total antibody, IgG or IGM.

4.5 Cytomegalovirus (CMV)

Introduction

Cytomegalovirus is a ubiquitous human viral pathogen. Human CMV is classified as a member of the herpes viruses ,are relatively large, enveloped DNA viruses that undergo a replicative cycle involving DNA expressions and nucleocepsid assembly within the nucleus

Although the herpes family produces diverse clinical diseases, the viruses shares the basic characteristics of being cell associated. These characteristics may play a role in the ability

of the virus to produce sub clinical infections that can be reactivated under appropriate stimuli. Dissemination of the virus may occur by oral, respiratory, or venereal route. It may also be transmitted preferably by organ transplantation or by transfusion of fresh blood.

Persistent infections characterized by periods of reactivation of CMV are frequently termed latent infections, although this condition has not been clearly defined for CMV. Acquired CMV infection is usually asymptomatic and can persist in the host as a chronic or latent infection. In most patients, CMV infection is asymptomatic, occasionally a self limited, heterophil negative, mononucleosis like syndrome results.

CMV infections is known to alter the immune system and produce overt manifestations of infection. Infection interferes with immune responsiveness in both normal and immunocompromised individuals. This diminished responsiveness results in a decreased proliferation response to the CMV antigen, which persists for several months. In patents with CMV monucleosis like syndrome, alterations of T. lymphocytes subsets result producing an increase in the absolute number of suppressor (CD) lymphocytes and a decrease in helper (CD) lymphocytes.

Serology

In cells infected by CMV, several antigens appear at varying times after infection. Before replication if viral DNA takes place, immediate early antigen and early antigens are present in the nuclei of infected cells.

Diagnostic Evaluation

Serologic methods to defect the presence of IgM antibodies can aid in the diagnosis of primary infection. Detection of CMV-specific IgM can represent primary infection or rare reactivation of infection.

Detection of significant increases in CMV- specific IgG antibody by methods such as complement fixation (CF), anti complement immunofluorescense (ACIF) and Enzyme Immunoassay (EIA) suggest, but do not prove recent infection or reactivation of latent infection. The EIA method for IgM and IgG, antibodies to CMV has replaced CF, ACIF, and IFA.

Latex particle agglutination and indirect hemogglutination are useful screening methods to obtain sero negative blood donors.

Newer CMV detection methods are being explored. CMV antigen detection in urine by EIA and cDNA is being developed. RNA transcript of CMV DNA is detectable in

peripheral blood mononuclear cells of sero positive individual by in situ hybridization (ISH) with DNA of CMV.

Review Questions

1. Explain the difference between screening and confirmatory tests for HIV,
2. Discuss test Algorithm in the diagnosis of HIV / AIDS
3. List what can be indentified by HIV testing technologies
4. Describe multi test algorithm for HIV antibody detection
5. List HIV tests that are used to indicate early infection and describe why they possess such quality
6. Describe the different generations of ELISA
7. Describe the principles of Indirect ELISA
8. State advantages of rapid tests in the diagnosis of HIV/ AIDS
9. Describe the HIV test algorithm in Ethiopia
10. Explain mode of transmission for different viral hepatitis
11. List common serological tests for viral hepatitis
12. Explain clinical manifestation of infectious mononucleosis (IMN)
13. Discuss heterophil antibodies in IMN
14. Describe diagnostic evaluation of Rubella infection.
15. Describe diagnostic evaluation of CMV.

CHAPTER FIVE
SEROLOGY OF RHEUMATOID FACTOR, SYSTEMIC LUPUS ERYTHETOMUS, C-REACTIVE PROTEIN, AND HUMAN CHORIONIC GONADOTROPIN HORMONE

5.1. Rheumatoid Factor (RF)

Rheumatoid factor belongs to a large family of antiglobulin usually defined as antibodies with specificity for antigen determinants on the Fc fragment of human or certain animal IgG. RF have been associated with three major immunoglobulin classes: IgM, IgG, and IgA. Of these IgM and IgG are the most common.

As indicated by its name, RF has particular application to diagnosis and monitoring of rheumatoid arthritis. Rheumatoid arthritis (RA) is a systemic syndrome in which chronic inflammation of the joints initiated by autoantibodies and

maintained by cellular inflammatory mechanisms is the major feature.

The autoantibodies are directed to self-immunoglobulin determinants. The formation of immune complexes in the joint spaces leads to activation of complement and destructive inflammation. The acute phase is followed by a delayed type hypersensitivity (DTH) type chronic inflammation. Chronic RA is believed to be driven by macrophages after initiation by DTH cells (TH1). The most common symptoms include a symmetric arthritis usually involving the small joint of the hands or feet and knees. Rheumatoid factor has been associated with some bacterial and viral infection (hepatitis and infectious mononucleosis) and some chronic infections (tuberculosis, parasitic disease, sub acute bacterial endocarditis, and cancer). Elevated values may also be observed in the normal elderly population.

5.1.1. Serologic tests

Tests for RA are designed to detect certain macroglobulin in the patient's serum that reacts with normal human IgG or normal animal IgG (i.e., rheumatoid factors).The majority of tests use particular carriers (i.e., erythrocytes, latex and bentonite particles) that transform the reaction between RF and IgG into visible aggregation. Basically, all the tests are designed to detect antibody to immunoglobulin. However, they

are not identical, because sometimes human and sometimes animal immunoglobulin are used as the coating for the particle. In some circumstances, the different tests give different results; therefore, one can postulate that a number of rheumatoid factors with different specificities are involved.

Included among the various serologic tests for the detection of RF are the latex fixation tests, the sheep cell agglutination test, the sensitized alligator erythrocyte test, bentonite flocculation test and the concanvalin A and complement fixation test for the detection of IgG RA factor. Of these tests (with the exception of the last, which is specific for IgG RF), the latex fixation and the sheep cell agglutination are the most popular. A number of preparations for use in rapid slide tests are also available.

5.2. Systemic lupus Erythematosus

Systemic lupus erythematosus (SLE) is the classic model of autoimmune disease. It is a systemic rheumatic disorder. Although no single cause of SLE has been identified, a primary defect in the regulation of the immune system is considered important in the pathogenesis of the disorder.

SLE is a disease of acute and chronic inflammation. The manifestation of SLE results from defects in the regulatory mechanism of the immune system. Lymphocyte subject

abnormalities are a major immunologic feature of SLE. The formation of lymphocytotoxic antibodies with a predominant specificity for T lymphocytes by SLE patients at least partially explains the interference with certain functional activities of T lymphocytes manifested by the patient with SLE.

Regulation of antibody production of B lymphocytes, ordinary function of the subpopulation of T suppressor cells, appears to be defective in SLE. Patients exhibit a state of spontaneous B lymphocyte hyperactivity with ensuing uncontrolled production of a wide variety of antibodies to both hosts and exogenous antigens.

Circulating immune complexes are the hallmark of SLE. Patients with SLE exhibit multiple serum antibodies that react with native or altered self antigens. Demonstrable antibodies include antibodies to nuclear components: cell surface and cytoplasmic antigen of polymorph nuclear and lymphocytic leukocytes, erythrocytes, platelets, and neuronal cells and IgG.

Laboratory evaluation of Antinuclear Antibodies (ANA)

The ANA method provides the laboratory with a simple and sensitive technique for detection and measurement of these antibodies. Indirect immunofiuorescence is the preferred initial screening procedure. If the ANA method is positive additional

immunologic evaluation is necessary to determine the specificity of the reaction. These evaluations include double immiunodiffusion, counter immunoelectrophoresis, passive hemaggiutination, enzyme linked immunosorbent assay, radioimmunoassay, and identification of nuclear antigens by immunoprecipatation or immunoblotting . These evaluations may demonstrate that more than one ANA specificity is present in the serum. LE cell preparation, however, has limited usefulness.

5.3. Acute-phase reactants/ Acute-phase Proteins

Acute-phase reactants are a heterogeneous group of serum proteins that have in common a rapid increase in concentration in the serum following acute tissue injury. They have a varied function in inflammation, but in general serve to increase or limit the damage caused by inflammatory mediators such as IL-1, TNF, INF-γ and INFβ-2

These cytokines stimulate production of acute phase reactants by the liver.

Some examples of acute-phase reactants proteins are:-

- C-reactive proteins(CRP)
- complement components (C2, C3, C4, C5, C9, factor B)
- Coagulation factors

They can have mediator function, inhibitor, scavenger, immune regulation and repair and resolution function.

5.3.1. C-reactive protein (CRP)

C-reactive protein is the most widely used indicator of an acute-phase response in man for the early indication of infections, inflammation or other diseases associated with tissue injury. Normally, the serum concentration of CRP is 0.1 mg/dl or less. After injury, rapid production in the liver results in concentrations as high as 100mg/dL. CRP is synthesized only in the liver, and synthesis is stimulated by IL- 6 and IL-1.

C-reactive protein got its name since it was first identified in the serum of patients with pneumonia where it precipitated with the C-polysaccharide on the pneumococcal cell wall.

Cleavage of CRP by enzymes from neutrophils produces fragment that promote chemotaxis and contain the tetra peptide called tufsin, which is also present in the CH2 domain of the immunoglobulin heavy chain. Thus, the biological effects of CRP are like those of immunoglobulin, including the ability to precipitate, to function as opsonin through binding to macrophages, and to fix complement.

Levels of CRP (increases 4 to 6 hours after tissue injury) parallel the course of the inflammatory responses and returns to lower undetectable levels as the inflammation subsides. It can increase as much as 100 fold in concentration in acute

inflammation and is the fastest responding and most sensitive indicator of acute inflammation. CRP increases faster than erythrocyte sedimentation rate in responding to inflammation, whereas the leukocyte count may remain within normal limits despite infection. An elevated CRP can signal infection many hours before it can be confirmed by culture results; therefore, CRP is the method of choice for screening for inflammatory and malignant organ disease and monitoring therapy in inflammation. Elevations of CRP occurs in nearly 70 disease states, including septicemia and meningitis in neonates, infections in immunosuppresed patients, burns complicated by infection, serious postoperative infections, myocardial infraction, malignant tumors, and rheumatic disease.

In general, the CRP is advocated as indicator of bacterial infection in at-risk patients in whom the clinical assessment of infection is difficult to make. However, a lack of specificity rules out CRP as a definitive diagnostic tool.

In clinical practice, CRP is particularly useful when serial measurement are performed. The course of the CRP level may be useful for monitoring the effect of treatment and for easy detection of post operative complications or internal infections. Note that half life of CRP is 5 to 7 hours. It falls much more rapidly than other acute phase proteins when patient recovers

Laboratory tests for C-reactive protein (CRP)

Rapid latex agglutination test

Principle

The agglutination test is based on the reaction between patient serum containing CRP as the antigen and the corresponding antihuman (CRP) antibody coated to the treated surface participles. The coated particles enhance the detection of an agglutination reaction when antigen is present in the serum.

Other test includes:-

- Complement fixation, not for routine clinical laboratory
- Fluorescent antibody
  Used to study binding of CRP to lymphocytes and their subpopulation
  Used primary as a research tools for localizing CRP in tissue.
- Precipitation
  Tube method
  Gel electrophoresis
- Laser nephelometry
  Sensitive, rapid and reproducible
  Can be used for large number of samples
  In brief, the procedure involves the measurement of light that is scattered by the insoluble immune

complexes in aliquot medium containing polyethylene glycol.

5.4. Serology of Human Chorionic Gonadotrophin (hCG) Hormone

5.4.1. Introduction

From the earliest stage of development (9 days old), the placenta produces hormones, either on its own or in conjunction with the fetus. The very young placental trophoblast produces appreciable amounts of a hormone, human chorionic gonadotropin (HCG) that is excreted in the urine. Human chorionic gonadotropin is not found in the urine of normal, young, nonpregnant woman.

As with all glylcoprotein hormones (LH, FSH, TSH), hCG is composed of two sub units, alpha and beta. The alpha sub unit is common to all glycoproteins and the beta sub unit confers unique specificity to the hormone. Because hCG is a glycoprotein hormone that is unique to the developing placenta (and some tumors), pregnancy tests are based on the detection of hCG in serum or urine. Its small size permits it to pass directly into the urine from the circulation.

Early in pregnancy, concentration of hCG in maternal serum rises quickly, with a doubling time of roughly two days during the first few weeks as the trphoblastic tissues increase in size.

Within 10 to 12 weeks, hCG values with peak at 150,000 to 200,000 mIU/ml and then gradually fall to normal plateau values of 10,000 to 50,000 mIU /ml in the second and 3rd trimesters.

A later- term pregnancy in which there is a sudden drop in hCG from the plateau may indicate threatened abortion. Ectopic pregnancies have much lower hCG values and do not go to term. Molar pregnancies and other trophoblastic malignancies can have very high values of hCG, considerably beyond those encountered in normal pregnancy.

Monoclonal based assay which uses two different antibodies, one against the α- subunit and one against the β sub unit in a sandwich that capture the whole hCG molecule on a solid phase is being developed.

Detection or quantitation of hCG is then generally accomplished by a color indicator reaction mediated by an enzyme (e.g., alkaline phosphatase) linked to the second antibody.

5.4.2. Serology of HCG in Urine

The amount of HCG excreted in the urine is almost the same as that found in the blood.

HCG can be detected in the urine of pregnant women 26 to 36 days after the first day of the last menstrual period or 8 to 10 days after conception. Pregnancy test should be negative 3 to 4 days after delivery. If the measured value in urine is negative, but clinical examination indicates possible pregnancy, the test should be repeated in two days. Urine pregnancy test may be negative even though serum tested at the same time is positive because the serum assay is more sensitive, being optimized for the protein matrix found in serum.

5.4.2.1. Urine pregnancy tests

Laboratory pregnancy tests are based on the detection of rapidly rising levels of hCG in urine. Immunologic pregnancy tests are done in one of two ways. They differ in the carrier for the external source of hCG, which is either a latex particle or red blood cells. The presence of hCG, is usually measured in urine because a urine sample is so easy to obtain.

Urine pregnancy testing kits can be divided in to:

- Rapid latex slide tests of the inhibition (indirect) or the direct type
- Haemagglutinaton inhibition

A. Inhibition (indirect) latex slide test

In this type of test, two reagents are supplied, an antiserum containing anti hCG antibody, and a latex reagent consisting

of polystyrene particles sensitized (coated) with hCG, positive and negative controls etc.

- In the inhibition test, urine is first mixed with the antiserum, and the latex reagent is added.
- If hCG is present in the urine, it will combine with the anti hCG antibody. This will leave no antibody free to combine with the latex hCG and therefore, there will be no agglutination of the latex particles.
- If there is no hCG in the urine, the antibody will be free to combine with the latex hCG and cause agglutination of the latex particles.
- In this test, therefore, no agglutination indicates a positive test and agglutination indicates negative test.

B. Direct latex slide test

Are more sensitive than inhibition tests.

In this test, the latex reagent consists of particles coated with the anti- hCG antibody. This reagent is mixed directly with the urine.

- If hCG is present in the urine, it will combine with the antibodies and cause agglutination of the latex particles.
- If no hCG is present in the urine, there will be no agglutination of the latex particles.

- In this test, therefore, agglutination of the particles indicates positive test and no agglutination indicates a negative test.

Note: the direct test is read in the opposite way to the inhibition (indirect) test.

C. Inhibition tube haemagglutination

In this type of test, the principle is the same as in the latex slide test except that the hCG is coated on red cells, not on polystyrene particles.

The urine is reacted with anti hCG antiserum in the small tube provided, and red cells coated with hCG are added. The contents of the tube are mixed and then left at room temperature (20-28 ^{O}C) for 1 ½ -2 hours to allow time for the red cells to settle.

- If the urine contains hCG, it will combine with the antibody. This will leave no antibody to react with the hCG on the red cells. The non-agglutinated cells will settle and be seen as a red ring in the bottom of the tube.
- If the urine contains no hCG, the anti hCG antibody will react with the hCG on the red cells and cause their agglutination (haemagglatination). The agglutinates will settle and be seen covering evenly the bottom of the tube.
- In the inhibition (indirect) haemagglutinatan tube test, therefore, a red ring of non-agglutinated cells in the bottom

of the tube indicates a positive test and a covering of agglutinated cells indicates a negative test.

N.B. Generally, urine for hCG is reported as positive for hCG if it is positive and negative for hCG if it is negative.
There are different ICT tests developed for pregnancy

D. Semiquantitative test

If required, the amount of hCG in specimen can be measured semiquantitatively by preparing serial dilutions of the specimen in physiological saline and testing each dilution.

- Most manufactures of slide and tube tests provide details of how to perform a semiqntitative technique. A more accurate result is obtained by using a tube technique.
- Quantitative analysis of hCG aids in making a differential diagnosis of a viable pregnancy versus a nonviable pregnancy, twins or multiple gestations, or developing hydatidiform mole.

5.4.2.2..Factors that affect urine pregnancy test

The time in the pregnancy when the test is carried out. Interfering substance (drugs , red cell etc) & sensitivity / specificity of the assay. Negative or inconclusive results may occur if the concentration of HCG in the urine is below that which the test is capable of detecting reliably.

The presence of excessive amounts of protein or blood in the urine may cause false positive results.

The presence of detergent can cause false positive or false negative result. Therefore, every material that will be used in this test should be free from detergent.

Turbid specimens (due to amorphous debris or epithelial cells) may give inconclusive results. Such specimens should be filtered or centrifuged.

Bacterial contamination of the urine may cause unreliable results. Heavily contaminated urine is unsuitable for testing.

Important- always read carefully the manufacturer's information leaflet.

5.4.2.3 Urine specimen

An early morning specimen is preferable because this is the most concentrated and will therefore, contain the highest level of HCG. If the specimen cannot be tested immediately, it should be refrigerated at 4^0c, but for not longer than 48 hours. Specimens preserved with boric acid are also suitable for testing. When tested, the urine (and test reagents) should be at room temperature.

Review Question

1. Describe the measurement of C-reactive proteins
2. List laboratory tests for C- reactive proteins (CRP)
3. State the application of rheumatoid factor testing
4. List the immunoglobulin classes that act as rheumatoid factor
5. List common serological tests of Rheumatoid factor.
6. Describe why serum or urine hCG determination is used as indicator of pregnancy
7. List serologic tests for detection of urine hCG and describe their principles
8. List factors that can affect serologic tests of urine hCG

CHAPTER SIX
SOME MISCELLANEOUS TECHNIQUES AND MONOCLONAL ANTIBODY PRODUCTION

Learning Objectives

At the end of this chapter, the students should be able to:

1. Describe the uses of isolation of lymphocyte populations
2. Describe and / or perform lymphocytes isolation procedures (Methods)

3. Describe methods of preparation of monoclonal antibodies
4. List uses of monoclonal antibodies

6.1. Miscellaneous technique

6.1.1. Isolation of Lymphocyte Populations

Introduction

In studies on humans, peripheral blood lymphocytes are most readily available sources of cells. Lymphocytes and their specific subpopulations can be isolated by: Fluorescent activated cell sorter (FACS), density gradient separation and rosetting.

Activities for which the lymphocytes can be separated could be:

- To detect the ability of a given B cell to produce a given antibody,
- To detect the ability of a given T cell to produce particular Cytokines,
- To test the ability of a given cell to be stimulated by a given mitogen.

The study of human T cells is best performed using purified cells, since the presence of other cell types may have indirect effects on T cell function. However, for any kind of functional assay on T cell specificity antigen - presenting cells are necessary.

A. peripheral blood Mononuclear Cells (PBMC) Isolation

The mononuclear cell fraction containing monocytes and lymphocytes is separated from polymorphonuclear cells and red blood cells by density gradient centrifugation.

Equipment and reagents

- Suppl. RPMI - 1640 medium: RPMI - 1640 (Life Technologies Inc, Gaithersburg, MD) contaning 2mML - glutanine (Biochrom seromed, Berline, Germany), 25mM N-(2 hydroxyethyl) piperazine -N-(2ethancesulfonic acid) (HEPES) (Biochrom), 100U ml penicillin, 100gml streptomycin (Pen-strep, Biochrom)
- Fetal calf serum (FCS) (e.g. Life Technologies Inc.) Which has been inactivated by heat (56^0c, 30 minutes) before use.
- Heat-inactivated human serum, blood group AB (HUS, obtained from a local blood transfusion center).
- Ficoll - Hypaque (Histopaque - 1077, sigma).
- 50 and 15ml conical centrifuge tubes (e.g. Greiner, Nurtingen Germany).
- Temperature controlled centeifuge with GH - 3.7 - horizontal rotor (e.g. heraeus or Beckman).
- Trypan blue, haemocytometer:

Procedure

- Peripheral blood is collected in sterile heparinized tubes. Heparinized whole blood (10ml) is mixed with 15ml suppl. RPMI - 1640.
- The mixture is carefully layered over 15ml of Ficoll - Hypaque in a 50-ml conical centrifuge tube.
- Spin for 20 min at 2000 rpm (900g at $4^{O}C$).
- The layer between Ficoll and the upper layer (containing RPMI - 1640 and serum) contains the mononuclear cell (MNC) fraction.
- Using a pipette remove 80% of the upper layer and recovers the interface (MNC) layer. Transfer the latter to a new 50-ml conical tube, and fill the tube with suppl. RPMI -1640/5% fetal calf serum (FCS) and centrifuge for 10min at 1300rpm (400g, $18^{0}c$).
- After the supernatant has been removed, the MNC pellet is resuspended in suppl. RPMI-1640/5% FCS, and washed twice. For the last wash 15ml conical tube can be used.
- Finally the cells are resuspended in 1ml suppl. RPMI - 1640/10% heat - inactivated human AB seum (HUS).

Counting and markers of cell death

Cell suspension (20ul) is diluted with 20ul 0.5% aqueous Trypan blue. The stained (dead) and non-stained (viable) cells are counted in a haemocytometer.

B. Separation of T and Non - T Cells from Monounclear Cells

The E - rosetting Technique

The E-rosetting technique describes a procedure for separating T cells and non -T cells from a population of MNCs (e.g. peripheral blood or synovial fluid MNCs). This method is based on the ability of human T cells to bind to sheep erthrocytes via their CD2 molecule. Neuraminidase treatment of sheep red blood cells (SRBCs) enhances the binding of SRBCs to T lymphocytes. First neuraminidase treated SRBCs are prepared. Secondly, SRBCs and MNCs are mixed to form rosettes (E^+, which are then isolated from the non - rosetting population (E^-, i.e., B cells and monocytes) by Ficoll gradient centrifugation. In the last step, bound SRBCs are separated from the rosetted T cells by hypotonic lysis.

Equipment and reagents for E - rosetting

- SRBCs (eg. From Biologische Arbeitsgemeinschaft Hessen. Germany): sterile PBS suppl, RPMI -1640 FCS, heat inactivated (Life Technologies inc.) Test - Neuraminidase (Centeon L.L.C., king of Prussia, PA): Ficoll density 1.09 (Biochrom).
- 15ml conical centrifuge tubes (e.g. Greiner or Falcon). Temperature controlled centrifuge (eg. Beckman or Heraeus).

Preparation of Neuraminidase - treated SRBC

A suspension of SRBCs (2ml) and sterile PBS (10ml) are placed in a 15ml conical centrifuge tube and spun at 2000 rpm (900g) for 10min, where after the PBS supernatant is removed and the cells are resuspended in PBS. This washing procedure is repeated twice. Before treatment with neuraminidase, washed SRBCs can be stored at 4^0C for 3 days. Part of the dry SRBC pellet (300µl) is incubated with 4.6ml RPMI -1640 and 100µl neuraminidase in a water bath (37^0C, 30 min), washed twice with RPMI -1640 (2000rpm. 10min), and finally resuspended in RPMI -1640 to a total volume of 5ml. The suspension is stored at 4^0Cuntil use.

Rosette formation and Ficoll density gradient centrifugation

1. MNCs are prepared by standard Ficoll - Hypaque centrifugation, washed, counted and suspended in suppl. RPMI - 1640/10% ($10x10^6$ cells mL^{-1}) The neuramindase - treated SRBCs are mixed with the MNCs (20 - 30 min, room temperature) to allow E-rosette formation, whereafter the mixture is layered over a Ficol solution (density 1.09) in a 15ml conical centrifuge tube. The volumes of SRBCs medium and Ficoll used in this protocol depnd on the number of MNCs to be separated. The tubes are centrifuged for 30 min at 2800 rpm.

2. Remove and decant about 80% of the upper layer (RPMI - 1640/ 10% HUS) from the centrifuged suspension. The E-rosette negative (monocytes/ B cell enriched) layer (E) is recovered from the interface layer with a pipette, transferred to a 15-ml conical tube, and washed with a pipette, transferred to a 15ml conical tube, and washed with suppl, RPMI - 1640/5% FCS.
3. The E-rosette-positive (T cell) pellet (E^+) is suspended in 1ml RPMI - 1640/10% FCS in the 15-ml tube. Cold distilled water (2ml) is added for hypotonic lysis of SRBCs and mixed gently. After a few seconds, add 8ml RPMI -1640/10% FCS. Transfer this suspension to a 50 - ml tube containing 40 ml RPMI -1640 / 10% FCS and centrifuge for 10min at 1300rpm.

C. Separation of T cell subsets

Purification of T - cell populations by indirect antibody panning

T cells expressing particular cell surface markers, such as the CD4, CD8, αß - TCR or TCR molecules can be selected by their capacity to bind to an antibody coated plastic plates. For example to purify $CD8^+$ T cells, isolated T cells (E^+ cells) are treated with a mouse anti - human monoclonal antibody against the CD4 molecule, and then incubated on plastic dishes that have been coated with an anti - mouse IgG

antibody. The T-cell populations that are not CD4 positive (i.e. the αßTCR $CD8^+$ and the γδTCR $CD8^+$ subpopulations), and do not therefore bind the mouse anti human CD4 antibody, will not adhere to the coated plate. These $CD4^-$ cells can be selected physically from the adherent $CD4^+$ subpopulation.

Equipment and reagents

- T-cell population (E^+ cells)
- Appropriate monoclonal antibody (eg. OKT4 or OKT8 hybridism supernatant containing anti -CD4 or anti -CD8 antibodies, or commercially available anti -CD4, anti -CD8 antibody); suppl. RPMI -1640; FCS, heat inactivated PBS, sterile
- Plastic six -well plates (Macroplate Standard Greine): 15ml conical centrifuge tubes (e.g. Falcon); sterile rubber scraper; temperature controlled centrifuge (e.g. Beckman or Heraeus).

I. Procedure for the separation of T cells into $CD4^+$ and $CD8^+$ T cells.

1. Preparation of the panning plate

   Goat anti-mouse Ig is diluted to 10µg ml^{-1} in suppl. RPMI -1640 and added to the wells of a plastic six -well plate (15ml per well). To Separate 2 x 10^6 to 3 x 10^6 T cells, one well of the panning plate is needed. Incubate overnight at 4^0C or for 60min at room temperature. Remove unbound Ig by using a sterile

pipette and gently wash the plate by adding 3ml PBS to each well (wash three times), Add 2ml suppl. RPMI - 1640 /5% FCS and keep the plate at 4^0C until the T cells are added to the plate (at least 30 min).

2. Pretreatment of the T cells. Prepare the monoclonal antibody (e.g. Sterile filtrated)

OKT4 Hybridoma supernatant containing these antibodies, or commercially available anti - CD4 antibodies diluted in sterile PBS at a concentration appropriate for flow cytometry according to the manufacture's instructions). Count the T cell population and place the cells in a 15 - ml centrifuge tube in suppl. RPMI - 1640/5% FCS; spin at 1300 rpm (400g) and 4^0C. Decant the supernatant and resuspend the cell pellet in 1 - 2 ml of the monoclonal antibody diluted in PBS. Incubate the tube containing the cells for 30min on ice (iced water) and then fill with suppl. RPMI - 1640/ 10% FCS. Centrifuge for **min** at 1300 rpm (400g) and 4^0C. After the supernatant has been removed, the cell pellet is resupended in suppl. RPMI-1640/10%FCS, and the wash repeated once. Finally the cells are resuspended in suppl. RPMI -1640 (1.5ml per $2x10^6$ to 3 x 10^6 cells).

3. Incubation the coated plate with the pretreated T cells: Remove the RPMI 1640 /5% FCS from the coated wells of the six well panning plate with a sterile pipette, and immediately add the pretreated T cells in

RPMI 1640 (1.5ml per well) spin the plate for 10min at 300 rpm and 4^0C carefully remove the plate from the centrifuge and incubate 30 min at 4^0c.

4. Collection of the negatively selected cells: Gently swirl the plates for 1 minutes and collect the supernatant containing the non - adherent cells using a sterile pipette. The negative selected, non adherent (i.e. CD4) T cells are washed twice with suppl. RPMi 1640 / 10% FCS in a 15ml conical tube counted and responded in supp. RPMI 16040/10% human serum or interleukin 2 supplemented medium depending on the culture proceeding this is non - adherent populations of cells should be 90 - 95% pure.
5. Collection of positively selected adherent $CD4^+$ T cells: Wash the plates gently with 3 ml suppl. RPMI - 1640/5% FCS per well (2-3 washes) until all non - adherent cells have been removed.

Pitfalls

The purity of the adherent cell population is greater than non-adherent population. However, it must be considered that the function of the adherent T cell population may be altered by the binding of specific antibodies to surface molecules to be positively selected.

II. Immunomagnetic Negative Selection of $CD4^+$ T cells

The protocol below is another cell separation techniques mediated by antibody-antigen reactions T cells (E^+ cells) are incubated with specific monoclonal antibodies to surface molecules (anti-CD8) to coat unwanted T cells. Magnetic beads coated with goat anti-mouse IgG are then applied to the cell suspension in order to bind the antibody coated cells. After binding; the target cells can be recovered using a strong magnetic field. Negative isolation is a method by which the $CD4^+$ subset is purified from the $CD8^*$ subset binding to the coated magnetic beads. Furthermore, in a positive selection step, the beads can be removed from the $CD8^+$ target cells by a process of detachment.

Equipment and reagents

- T -Cell population (E^+celles)
- Appropriate monoclonal antibody (e.g anti CD8 antibody by **ptarmigan**) goat anti-mouse IgG coated magnetic beads (Dynabeads M-450, Dynal Oslo, Norway). Sterile PBS; FCS, heat inactivated coating medium (Hanks balanced salt solution (HBSS) without Ca^{2+} , $Mg2^+$ or phenol Red, supplemented with 10% FCS 20mM HEPES). Suppl. RPMI - 1640 HUS heat inactivated.
- Magnetic separation device (Dynal MPC-1) mixing device (Dynal MX1. 2 or 3) 15ml centrifugation tubes

(e.g. Falcon) vortex mixer, temperature controlled centrifuge (e.g. Beckman or Heraeus)

Procedure

All steps in the protocol are done at 4^0C.

1. Prewash of Dynabeads M-450: transfer the required number of Dynabeads M-450 from the vial to a polypropylene washing tube containing PBS/2% FCS (washing buffer), and place on the Dynal MPC -1 for 2 min, decant the supernatant, resuspend in excess washing buffer, and replace on the Dynal MPC -1. Finally, resuspend in a small volume of coating medium (e.g the volume originally pipette from the vial).
2. Antibody coating of $CD8^+$ T cells: Resuspend washed T cells in 10ml coating medium at 2 x 10 cells mL^{-1} in a 15ml conical tube and add 1ml anti-CD8 monoclonal antibody at a 10x saturating concentration. Incubate for 30 min at 4^0C with gentle tilting and rotation (e.g. in a mixing device).
3. Wash twice in coating buffer (centrifugation at 1000 rpm, 4^0C) to remove unbound antibody.
4. Add the suspension of washed Dynabeads and incubate for 30min at 4^0C with gentle tilting and rotation (e.g. in the mixing device) to keep cells and beads in suspension.

5. Place the tube in Dynal MPC and leave it to rest for 2 minutes to magnetically remove the CD8, cells labeled by antibody and coated with beads. Transfer the unbound cells to a fresh tube, perform a second magnetic separation, count, and resuspend in suppl. RPMI 1640/10% HUS. Negatively selected cells obtained by this method are unstipulated, pure and of high yield.
6. For recovery of positively selected $CD8^+$ cells, remove the tube from the Dynal MPC, and wash the resettled cells by resuspending in RPMI-1640/10% HUS. Repeat step 5 twice. These positively selected cells can be removed from the beads by a process of detachment.

III. Rosette Test

Subsets of T lymphocytes can be identified by their differing membrane structures called markers. Markers are categorized as antigen and receptors and can be detected by rosette technique. The E rosette forming cells were assigned to T cell lineage and the E-rosettes become the principle marker for identification and enumeration of human T cells. The presence of FC receptors for IgG or IgM on T lymphocyte has been correlated with their functional activity. Cells with IgM receptors were shown to provide help for B cell differentiation

to plasma cell, whereas cells with IgG receptors were reported to function as suppressors.

1. E-ROSETTE TEST

Spontaneous rosette formation with untreated sheep erythrocytes was performed with some modification.

- Separate Lymphocytes and adjust the count to $2.5x10^6$ / ml in PBS.
- Prepare 1% sheep erythrocyte suspension in PBS after 3 times washing in PBS.
- Then 50 micro liters of bovine serum albumin will be taken in tube in which 100μl of lymphocytes suspension and 100ul of 1% sheep RBC suspension will be added.
- Then centrifuge for 5 minutes at 1000rpm
- After incubation at 4^0C for 1hour, 0.1% toludine will be added and rosette-forming cells will be counted.

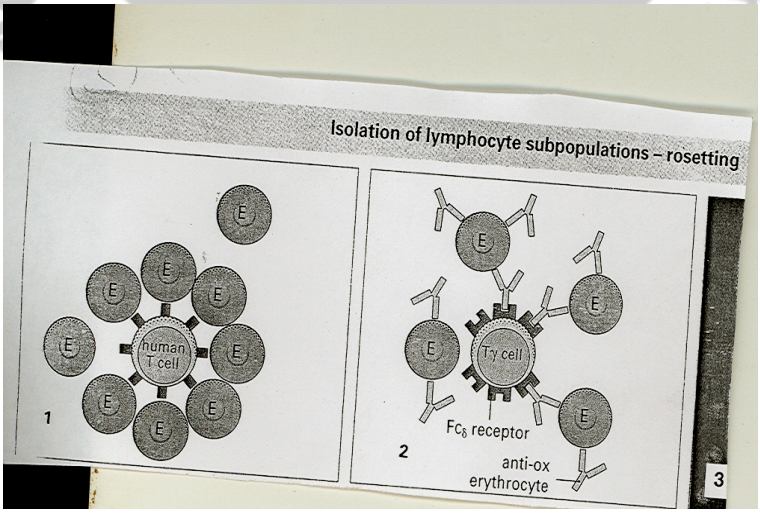


FIG. 6.1 E-rosetting

2. IgM and IgG Rosette test

Step 1: Preparation of anti-ox antibody

- Collect blood from an ox by vein puncture from external jugular vein.
- Separate plasma and cells by centrifuging under sterile condition.
- Inject 1ml of sterile packed Ox RBC intraperitoneally into rabbit.
- After 10 days, bleed the rabbit and test serum for anti-ox IgG, Ig A and IgM antibodies.

Step 2: preparation of Anti-Ox IgG and IgM sensitized cells

- Suspend 0.5ml of 50% washed red cells separately in 2.5ml of IgG and IgM fraction (IgG and IgM fraction obtained by column chromatography)
- Keep the mixture at 37^0C for 1 hour for IgG sensitization and at 4^0C for 1 hour for IgM sensitization
- Wash cells 3 times with PBS and prepare 1% suspension.

Step 3: Running IgM/IgG Rosette test

- Mix 100μl patient T lymphocytes (separated by nylon wool) with 100μl of 1% IgG and IgM sensitized Ox cells in different precipitin tubes.
- Centrifuge for 5 minutes at 1000 rpm and incubate for 30 minutes at 37^0C for the IgG rosette and 4^0C for IgM rosette.
- Add toludine blue dye (0.1%) and count the percentage of rosette forming cells under high power field using ordinary microscope.

IV. Cell sorting in the fluorescence activated cell sorter (FACS).

A suspension of cells is allowed to react with antibodies that are specific for particular molecules on the surface of one of the cell types in the mixture. The antibody has a fluorochrome attached to it. The suspension is then mixed with a buffer (sheath fluid) and droplets, each containing a single cell, are generated by ultrasonic vibrations in a nozzle. The droplets pass one by one through a laser beam, a beam of high intensity light of a particular wavelength. As the beam hits the cell, two things happen. The fluorochrome molecules absorb the light, but emit light of another wavelength. The emitted light is focused by collecting lenses on a barrier filter, which only allows light of a certain wavelength to pass through. Light detectors (photomultipliers) placed behind the barrier filter can then record whether light of a given wavelength has been

emitted from the cell and passed through the filter. At the same time, however, because of the cell curvature and surface unevenness, the light of the laser beam hits the cell at different angles and in turn is reflected from the cell at different angles, i.e. it is scattered. The character of the light scatter depends on the cell's size and density; the larger and denser the cell, the more light it scatters. The degree of light scatter is estimated by measuring light rays reaching the photomultipliers at two different angles in relation to the laser beam; a low angle (a forward scatter) and a right or obtuse angle (side scatter). The computer then uses these two estimates to determine the size and density of the cell. Based on this information and information regarding the emission of the fluorescent light, the computer checks whether the cell meets certain criteria for a particular cell type and, depending on the outcome, sends a signal to impart a certain electric charge to the droplet. As the droplets pass through an electric field generated by the deflection plates, they are sorted according to their charge and collected in tube.

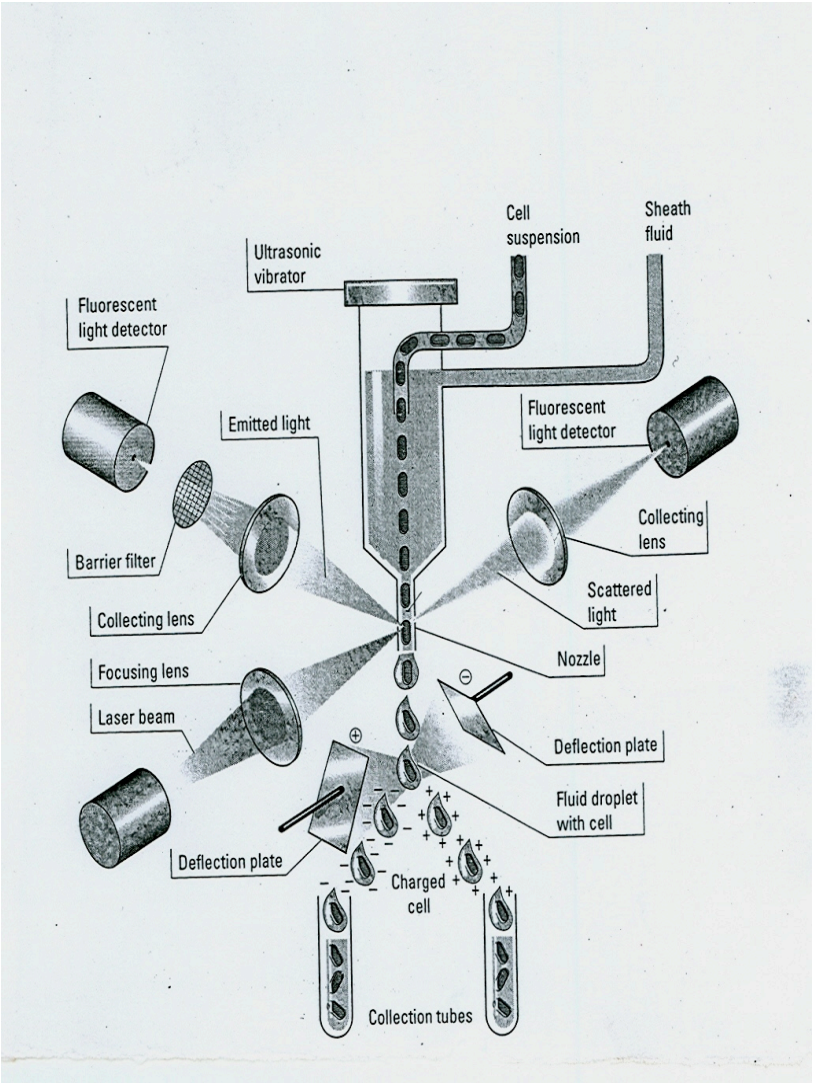


Fig. 6.2 Cell sorting in the fluorescence activated cell sorter

6.2. Methods of Monoclonal Antibody Production

6.2.1. Hybridoma Technique

Large quantities of absolutely pure, specific immunoglobulin directed against an antigen of interest can be produced by fusing a normal plasma cell making the antibody of interest with a myeloma cell with the capacity for prolonged growth in tissue culture. The resulting mixed cell is called hybridoma.

The first stage in making a hybridoma is to generate antibody producing plasma cells. This is done by immunizing a mouse against the antigen of interest and repeating the process several times to ensure that it mounts a good response. Two to four days after administration of antigen, the mouse's spleen is removed and broken up to form a cell suspension. These spleen cells are suspended in culture medium together with a special mouse myeloma cell line. It is usual to use myeloma cells that do not secrete immunoglobulins since this simplifies purification later on. Spleen cells are fused with a myeloma cell line by the addition of polyethylene glycol (PEG) which promotes membrane fusion. Only a small proportion of the cells fuse successfully. The fusion mixture is then set up in culture with medium containing 'HAT'. HAT is a mixture of hypoxanthine, aminopterin and thymidine.

There are two biosynthetic pathways by which cells can produce nucleotides and hence nucleic acids. The myeloma

cells are selected so that they lack the enzyme hypoxanthine phosphoribosyltransferase and as a result cannot utilize hypoxanthine in the culture medium to produce inosine, a pryimidine precursor. They are obliged to utilize an alternative biosynthetic pathway involving thymidine. But the aminopterin in the culture is a drug that prevents myeloma cells from making their own thymidine. Since the myeloma cells cannot use hypoxanthine and the aminopetrine stops them from using the alternative synthetic pathway, they cannot make nucleic acids and will soon die. Hybrids made from a myeloma and a normal cell will grow; they possess hypoxanthine phosphoribosyl transferase and can therefore use the hypoxanthine and thymidine in the culture medium and survive. The spleen cells die in culture naturally after 1-2 weeks. Any wells containing growing cells are tested for the production of the desired antibodies (using RIA or ELISAs) and if positive, the cultures are cloned by plating out so that there is only one cell in each well. This produces a clone of cells derived from a single progenitor, which is both immortal and producer of monoclonal antibody.

6.2.2. Recombinant DNA techniques

- Attempts are also being made to replace altogether the hybridoma method by recombinant DNA techniques.
- One such attempt focuses on the gene segments that specify the fragment antigen binding (Fab) of an immunoglobulin molecule, the V_H C_{H1} and V_LC_L.
- These segments can be amplified by PCR from many different mRNA (cDNA) molecules expressed in a population of cells undergoing an immune response. The amplified segments are inserted into a suitable vector, cloned and paired randomly (always one, V_H C_{H1} with V_LC_L, in a suitable vector) and the pairs translated into proteins (Fabs).
- Screening of this combinatorial library of antibodies with labeled antigen then identifies these combinations that bind this antigen.
- The identified V_HC_{H1}-V_LC_L pairs are placed in to an expression vector, either bacterial or mammalian, and used to produce large quantities of antibodies with selected specificity.

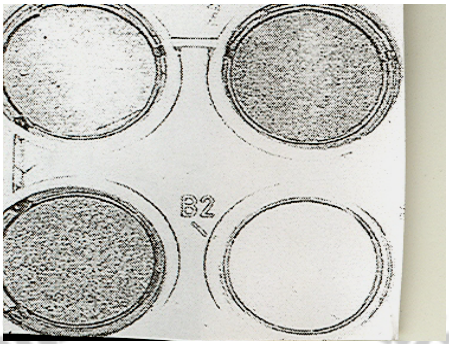
Uses of monoclonal antibodies

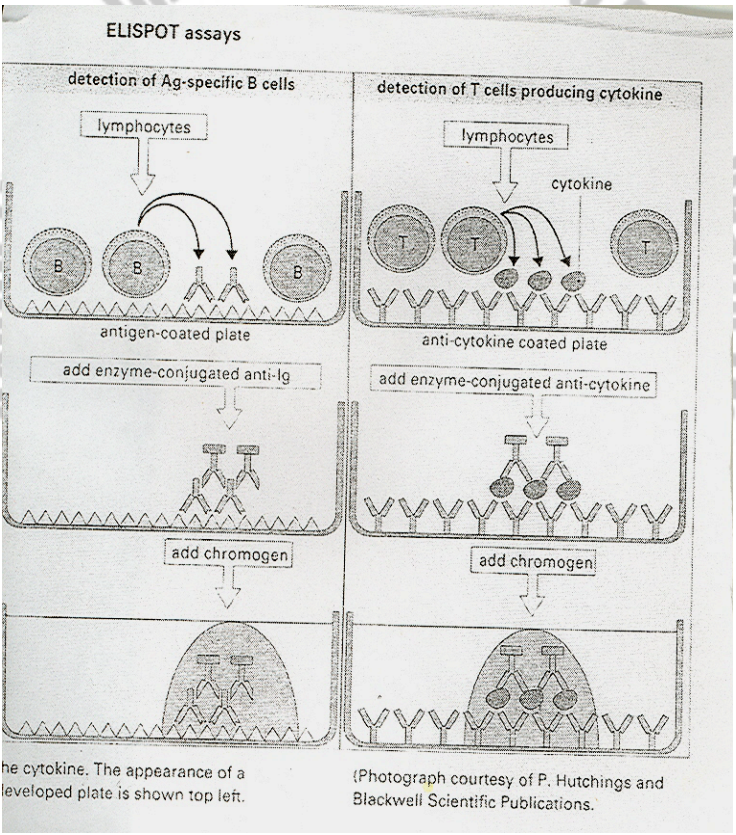
- The greatest impact of Monoclonal antibodies in immunology has been on the analysis of cell membrane antigens.
- Because Monoclonal antibodies have a single specificity compared to the range of antibody molecules present in the serum, monoclonal antibodies have multiple clinical applications including:
    - Identifying and quantifying hormones
    - Typing tissues and blood
    - Identifying infectious agents
    - Identifying clusters of differentiation for the classification and follow-up therapy of leukemias and lymphomas
    - Identifying tumor antigens and autoantibodies
    - Immunotherapy

6.3.Effector-Cell Assay

Various methods have been developed for assaying lymphocyte-effector functions, including antibody production, cytotoxicity, and T-cell mediated help and suppression. Individual B cells producing specific antibody or individual T cells secreting particular cytokines may be detected by ELISPOT assay. For detection of antibody –producing cells, the lymphocytes are plated onto an antigen-sensitized plate. Secreted antibody binds antigen in the immediate vicinity of cells producing specific antibody. The spots of bound antibody

are then detected chromatographically using enzyme coupled to anti-immunoglobulin and a chromogen. For detection of cytokine-producing cells, the plates are coated with anti-cytokine and the captured cytokine is detected with enzyme coupled antibody to a different epitope on the cytokine. The appearance of a developed plate is shown on top left of Fig. 6.





he cytokine. The appearance of a
leveloped plate is shown top left.

(Photograph courtesy of P. Hutchings and Blackwell Scientific Publications.

Fig. 6.3 ELISPOT assays

Review Question

1. State the use of isolation of lymphocyte populations.
2. List and describe the mechanisms by which lymphocytes and their specific subpopulations can be isolated.
3. List and describe methods of monoclonal antibody production.
4. List uses of Monoclonal Antibodies.

GLOSSARY

Acute phase proteins - plasma proteins whose concentration increases or decrease during inflammation.

Antibody - A globulin formed in response to exposure to an antigen; an immunoglobulin.

Antigen - A macromolecule that, when introduced into a foreign circulation, will induce the formation of immunoglobulins or sensitized cells that react specifically with that antigen.

Antigen Determinant sites - Unique portions of the structure of an antigen that are responsible for its activity.

Anti-Human Globulin - An antibody preparation that contains antibody to a range of globulins (polyspecific) or to a single globulin (monospecific) used to setect sensitized particles.

Antiserum - A serum containing antibodies

Antistreptolysin O (ASO) - An antibody produced against streptolysin O, a hemolysin produced by streptococci (particularly group A).

Agglutination - The aggregation or clumping of cellular or particulate antigens by an antiserum containing antibodies to one or more of the surface antigens.

C-Reactive protein - An acute phase protein produced by the liver in early inflammatory response, which is capable of precipitating the C- polysaccharide extract of pneumococcus.

Carrier - A molecule that, when coupled to a hapten, renders the hapten immunogenic.

CD4 - The protein receptor on the surface of a target cell to which the gp 120 protein of the HIV viral envelope binds.

Complement - A humoral mechanism of nonspecific immune response consisting of at least 14 components that proceed in a cascading sequence of activation resulting in cell lysis.

Complement Fixation - The fixation (or binding) of complement in a reaction with antigen and antibody.

Cross-Reactive Antigen - An antigen so structurally similar to a second antigen that it will react with antibody to the second antigen.

Delayed Hypersensitivity - Type IV hypersensitivity mediated by lymphokines released from sensitized T lymphocytes.

Direct Agglutination - An agglutination reaction that occurs through the direct combination of antigen and antibody.

Electrophoresis (of serum) - The separation of serum proteins according to their rate of travel when an electric current is passed through a buffer solution. The supporting medium can be whatman paper, starch, or agar gels.

Enzyme –Linked Immunosorbent Assay (ELISA) - A serologic test in which one of the reagents is labeled with an enzyme.

Fc Fragment - The fragment of the antibody molecule that, in certain species can be crystallized. It consists of two pieces of heavy chain.

Febrile Disease - A disease characterized by high fever.

Fluorescence - A form of luminescence in which a molecule absorbs light energy of one wavelength and emits light energy of a lower wavelength in less than 10^{-4} seconds.

Fluorescent Microscope - A modified dark field microscope that separates excitation wavelengths from emission wavelengths.

Fluorochrome - An organic compound that fluoresces when exposed to short wavelengths of light and that is used to label an antibody so that it can be visualized.

Hemagglutination - The agglutination (or clumping) or red blood cells, especially by antiserum.

Hemagglutination Inhibition Technique - The technique used for the detection of antibodies which involves the blocking of agglutination of erythrocytes.

Hemolysin - An antibody that in cooperation with serum complement will cause the hemolysis of erythrocytes.

Hemolysis - The lysis of red blood cells by specific antibody and serum complement.

Hepatitis - Inflammation of the liver caused by a virus or other agent (e.g. drugs).

Heterophil Antibody - An antibody produced in response to one antigen that will react with a second genetically unrelated antigen. Sometimes spelled “heterophile”

IgG - The most abundant immunoglobulin in serum; it is predominant in immunity against bacteria and viruses and is the only immunogloebulin to cross the placenta.

IgM - An immunoglobulin that is usually produced first in response to antigen challenge. It is the third most abundant in serum.

Immune Complex - A complex of antigen with antibody (which may involve complement) that can be soluble or can deposit in tissues.

Inflammation - Tissue reaction (redness, tenderness, pain, swelling) to injury by physical or chemical agents, including microorganisms.

Inhibition - The prevention of a normal reaction between an antigen and its corresponding antibody, usually because an antigen of the same specificity but from another source is present in the serum.

Lysis - The irreversible leakage of cell contents following membranes damage.

Optimal proportions - The point of dilution in a serologic reaction that gives a positive reaction.

Plasma - The fluid component of blood plasma Cell A cell 10 to 20µm in diameter that can activity synthesize immunoglobulins and can be distinguished morphologically from similar cells.

Postzone - The failure of a serologic reaction to occur in extreme dilutions of the antibody.

Prozone - The failure of a serologic reaction to occur in high concentration of the antibody

Radioimmunoassay - An immunologic test using radiolabeled antigen, antibody, complement, or other reactions.

Radioisotope - An atom with an unstable nucleus that spontaneously emits radiation as it decays to a stable nucleus.

Reagin

Receptor - A cell surface molecule that binds specifically to particular proteins or peptides in the fluid phase.

Seroconverstion - The detection of specific antibody in the serum of an individual in whom the antibody was previously undetectable.

Serum - The fluid portion of the blood after the blood clots.

Shared Antigen - A cross-reactive antigen (i.e. one that will react with an antibody induced by some other antigen).

Specificity - The special affinity between an antigen and its corresponding antibody.

Titer - The greatest dilution of a substance used in a serologic reaction that will produce the desired result.

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