LABORATORY DIAGNOSTIC

Subjects: Immunology
Study programme: General Medicine

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Academic year: 2015/2016
Department of Immunology

Laboratory of Cellular Immunology
Flow Cytometry Laboratory
Immunochemistry Laboratory
Tissue Culture Laboratory
Advances in diagnostic immunology are largely driven by instrumentation, automation, and the implementation of less complex and more standardized procedures.

- Miniaturization
- Amplified immunoassays
- Flow cytometry
- Molecular methods
Assumptions of rational diagnostics

- The broad spectrum of immunoassay methods
- Knowledge of the clinical value of immunological tests
- Rational indication of immunological tests
- A comprehensive assessment of results with clinical conditions
Determination of Immunological Parameters

- Acute stage of the disease
  - Screening
  - Treatment monitoring

- Autoimmunity
- Inflammation
- Hypersensitivity
- Allergy

- Normal function
- Deficiency or functional insufficiency
- Hyperactivity or inappropriate activity
Examination of cell-mediated immunity

- Analysis of living cells
- Correct collection to tube with anticoagulants - EDTA, heparin
- Sample storage at room temperature
- Fast transport of material to the laboratory

- Blood
- BAL
- Cerebrospinal fluid
- Synovial fluid
Examination of humoral immunity

- Serological assays
- Sample storage at $-80\,^\circ C$, $-20\,^\circ C$
- Serum
- Plasma
### Cellular immune mechanisms

- Leukocyte count and differential leukogram
- Examination of lymphocyte subpopulations
- Expression of adhesion molecules
- The functional activity of T and B cells, NK cells
- Neutrophil function, basophils, eosinophils, monocytes / macrophages
Humoral immune mechanism

- Detection of plasma proteins
- Immunoglobulins IgG, IgM, IgA, IgE
- Acute-phase proteins (CRP, alfa-1-antitrypsin, alfa-2-macroglobulin, orosomucoid, haptoglobin, ceruloplasmin)
- Complement system
- Cryoglobulins
- Immune complexes
- Specific antibodies IgE, IgA, IgG
- Autoantibodies (ANA, anti-ds-DNA, anti-DNP, ANCA, AMA,...)
Laboratory Information System

A laboratory information system (LIS) is a software system that records, manages, and stores data for clinical laboratories.

Functions of LIS

- patient management, including admission date, admitting physician, ordering department, specimen type, etc.
- patient data tracking
- test ordering
- quality assurance
- workload and management reporting
- workflow management
- billing
Principles of immunological assays - serological and cellular methods

ANTIGEN – ANTIBODY INTERACTION

Ag-Ab Reactions: applications in laboratory diagnosis
Definition of terms

**Antigen** = foreign substance that, when introduced into the body, is capable of stimulating an immune reaction e.g. foreign molecules in bacteria, viruses, protozoa, serum components, etc.

**Antibody** (immunoglobulin) = large protein produced by plasmocytes which identifies and neutralises antigens

**Immune reaction** = reversible binding of antigen to homologous antibody (high specificity)
### Laboratory Methods of Clinical Immunology

#### White Blood Cell Counts and Differential

<table>
<thead>
<tr>
<th>Microscopy – Blood smear</th>
<th>Hematological analyzer</th>
<th>Flow cytometry</th>
</tr>
</thead>
</table>


HEMATOLOGICAL ANALYSIS

COMPLETE BLOOD COUNT (CBC)

WHITE BLOOD CELL (WBC) DIFFERENTIAL
HEMATOLOGICAL ANALYSIS

(a) Neutrophil: Multilobed nucleus, pale red and blue cytoplasmic granules
(b) Eosinophil: Bilobed nucleus, red cytoplasmic granules
(c) Basophil: Bilobed nucleus, purplish-black cytoplasmic granules
(d) Lymphocyte (small): Large spherical nucleus, thin rim of pale blue cytoplasm
(e) Monocyte: Kidney-shaped nucleus, abundant pale blue cytoplasm
HAEMATOLOGICAL SYSTEM
Proven Technology

The proven technologies – Fluorescent Flow Cytometry, Hydrodynamic Focusing and Non-cyanide Hemoglobin

Direct current Impedance
WBC Differentiation

The combination of side scatter (cell complexity), forward scatter (size) of nucleated cells provides a concise and precise image of each detected peripheral blood cell.

This 3-dimensional blood cell analysis provides unique accuracy and precision. Fluorescence labeling of peripheral blood cells is a milestone for the routine leukocyte differential.
Whole Blood Reportable Parameters

WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, NEUT%, LYMPH%, MONO%, EO%, BASO%, NEUT#, LYMPH#, MONO#, EO#, BASO#, RDW-SD, RDW-CV, MPV
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>White Blood Cell (leukocyte) Count</td>
<td>RBC</td>
<td>Red Blood Cell (erythrocyte) count</td>
</tr>
<tr>
<td>NE%</td>
<td>Neutrophil percent</td>
<td>Hgb</td>
<td>Hemoglobin concentration</td>
</tr>
<tr>
<td>NE#</td>
<td>Neutrophil number</td>
<td>Hct</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>LY%</td>
<td>Lymphocyte percent</td>
<td>MCV</td>
<td>Mean Corpuscular Volume</td>
</tr>
<tr>
<td>LY#</td>
<td>Lymphocyte number</td>
<td>MCH</td>
<td>Mean Corpuscular Hemoglobin</td>
</tr>
<tr>
<td>MO%</td>
<td>Monocyte percent</td>
<td>MCHC</td>
<td>Mean Corpuscular Hemoglobin Concentration</td>
</tr>
<tr>
<td>MO#</td>
<td>Monocyte number</td>
<td>RDW</td>
<td>Red Cell Distribution Width</td>
</tr>
<tr>
<td>EO%</td>
<td>Eosinophil percent</td>
<td>Plt</td>
<td>Platelet count</td>
</tr>
<tr>
<td>EO#</td>
<td>Eosinophil number</td>
<td>MPV</td>
<td>Mean Platelet Volume</td>
</tr>
<tr>
<td>BA%</td>
<td>Basophil percent</td>
<td>PDW</td>
<td>Platelet Distribution Width</td>
</tr>
<tr>
<td>BA#</td>
<td>Basophil number</td>
<td>Pct</td>
<td>Plateletcrit</td>
</tr>
<tr>
<td>RET%</td>
<td>Reticulocyte percent</td>
<td>RET#</td>
<td>Reticulocyte number</td>
</tr>
</tbody>
</table>
**Differential-Related**

The discriminant function (DF) 1 scatterplot, Figure , shows lymphocyte 4, monocyte 1, neutrophil 2, and eosinophil 3 populations. The basophil population is behind the upper right quadrant of the lymphocyte 4 population. For purposes of the display, the axes are labeled: Volume and DF 1. DF 1 is derived primarily from the light scatter measurement. Volume is determined by the low-frequency impedance measurement.
Lymphocyte Subpopulations

- E-rosette test
  - Isolation of Lymphocytes
- Fluorescence Microscopy
- Flow Cytometry
Isolation of Lymphocytes
FLOW CYTOMETRY

- is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second

- is routinely used in the diagnosis of health disorders, especially blood cancer, immunologic disorder, and has many other applications in basic research, clinical practice and clinical trials
FLOW CYTOMETRY
Hydrodynamic focusing
Identification of immune cell subsets

CD45+ Leukocytes

CD14 monocytes/macrophages

CD3+ CD3+CD4+ (Th) CD3+CD8+ (Tc) T-lymphocytes

CD3+HLADR+ activated T-Ly

CD19+ B-lymphocytes

CD3-CD(56+16)+ NK cells
Surface adhesion molecules

**Immunoglobulin superfamily**
- ICAM-1/CD54
- ICAM-2/CD102
- ICAM-3/CD50
- VCAM-1/CD106

**Selectins**
- E-selectin/CD62E
- P-selectin/CD62P
- L-selectin/CD62L

**Integrins**
- LFA-1...CD11a/CD18
- VLA-4...CD49d/CD29
Importance of Immunophenotyping

- Diagnosis and classification of primary and secondary immunodeficiency
- Classification of leukemias and lymphomas
- Monitoring of immunotherapy and chemotherapy in immunodeficient conditions and malignant diseases
- Diagnosis and monitoring of autoimmune diseases
- Monitoring of immunity after organ transplantation
- Assessment of immune status in HIV infection
## Laboratory Methods of Clinical Immunology

Function tests of Lymphocytes and NK cells

<table>
<thead>
<tr>
<th>T lymphocyte activation</th>
<th>B lymphocyte function</th>
<th>NK cell function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte Transformation Testing - LTT</td>
<td>Secretion of Immunoglobulins</td>
<td>Cytotoxicity Assay</td>
</tr>
</tbody>
</table>
The importance of lymphocyte function tests

- Diagnostic and monitoring of the immunological profile of primary and secondary immunodeficiency
- Determination of lymphocyte immunocompetence and lymphocyte subpopulations disorders
- Monitoring of T lymphocytes immunoregulatory role
- Immunomodulatory therapy monitoring and study of the drugs influence
- The detection of hypersensitivity to a specific antigen (LTT – drugs – type IV allergy)
### Function tests of Granulocytes

<table>
<thead>
<tr>
<th>Phagocytic activity %</th>
<th>Oxidative burst</th>
<th>Cytotoxic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytic index</td>
<td>Chemiluminiscence</td>
<td>Microscopy</td>
</tr>
<tr>
<td></td>
<td>Flow Cytometry</td>
<td>Flow Cytometry</td>
</tr>
</tbody>
</table>
DETECTION OF PHAGOCYTOSIS

Phagocytosis – tests *in vitro* – measurement of ingested material by phagocytic cell (Mo/Ma, Ne, Eo)

Substrate: erythrocytes, E.coli, zymozan (yeast), various particles

Quantification: colorimetric ELISA method or by microscopy or flow cytometry
The importance of granulocyte function tests

Diagnostics of primary / secondary deficiencies in phagocytosis
Monitoring of functional and metabolic disorders of phagocytes
What is Cell Culture?

- Removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment.
TISSUE CULTURE

- Neutralization tests (e.g. viruses)
- Cytotoxicity assays (Tc, NK)
The effects of a toxin on a susceptible cell and neutralization of the toxin by antitoxin
Cytokines secretion by T cells - ELISPOT

- **a** T cells (at known concentration) with specific antigen in cell-culture well
- **b** ELISPOT wells are pre-coated with anti-cytokine antibody (Ig)
- **c** Transfer of T cells to ELISPOT plate
- **d** Incubation of cells; secretion of cytokine
- **e** Ig bound to nitrocellulose
- **f** Washing off of cells; coating of plates with secondary Ig
- **g** Cytokine binds to Ig
- **h** Washing of plate; addition of substrate; washing of plate
- **i** Enzyme-coupled secondary Ig binds to cytokine
- **j** Insoluble coloured product

ELISPOT assay to quantify secretion of cytokines by T lymphocytes (T cells)

*Expert Reviews in Molecular Medicine*
Cross-match

Evolution of HLA Antibody Detection

Cytotoxicity | Enhanced Cytotoxicity | Flow Cytometry

Anti-HLA Antibody → Ly → C1 → Membrane Attack Complex → Dye → Flow Cytometer

Enhanced Cytotoxicity

Anti-HLA Antibody → Ly → Anti-Human Globulin → Membrane Attack Complex → Dye → Flow Cytometer

Flow Cytometry

Anti-HLA Antibody → Ly → Flourescenated Anti-Human Globulin → Membrane Attack Complex → CD19 or CD3 → Flow Cytometer

TEST determine compatibility between a donor and recipient in organ transplantation - recipient serum is tested against donor cells
Immunochemical methods

Antigen-antibodies reactions,
Serological methods, Precipitation methods,
Enzyme Immunomethods, Immunoanalysis
with various particles, Electrophoresis methods,
Immunoaffinity methods,...
Serological reaction

Ag + Ab

Specific
Non-specific

Qualitative
Quantitative
Methods for Ag-Ab detection

- Precipitation
- Agglutination
- Hemagglutination and Hemagglutination inhibition
- Viral neutralization test
- Radio-immunoassays
- ELISA
- Immunofluorescence
- Immunoblotting
- Immunochromatography

Enzyme-linked Immunosorbent Assay
Agglutination: on slide/in tubes

- clumping together by antibodies of microscopic foreign particles:
  - red blood cells
  - bacteria
  - inert particles (latex)
- agglutinated particles are visible with the naked eye (pellet-like agglutination product)
Antigens (soluble)

Zone of equivalence: visible precipitate

Antibodies

Precipitation band
Agglutination and Precipitation

Agglutination

Precipitation

Bacterium

Soluble molecule
Precipitation tests

- Agar gel immunodiffusion tests “Double immunodiffusion” (AGID)
- Single radial immunodiffusion (SRID)
- Immunoelectrophoresis (IE)
- Immunochromatography
Radial Immunodiffusion

Radial Immunodiffusion, a variation of the agar precipitation technique, is used in clinical immunology for the detection and quantitation of all classes of Immunoglobulin's, complement, and other serum components.
What is Electrophoresis?

Electrophoresis is a laboratory technique for separating molecules based on their charge.
**Immunolectrophoresis**

Migration of molecules due to electric charge

Positive particles travel to cathode ($-$)

Negative particles travel to anode ($+$)

Precipitin
GEL ELECTROPHORESIS

The separation of DNA, RNA or proteins mixtures according to molecular size
ELECTROPHORESIS
Separation of proteins on a polyacrylamide gel

Vertical Electrophoresis System

Horizontal Electrophoresis System
Autoradiographic detection
Workflow

Sample Preparation

Electrophoresis

Transfer

Fluorescent detection

FieldBrite™ XT Technology
Transfer of proteins onto nitrocellulose
Affinity Chromatography

Fig. Affinity chromatography uses antigen-antibody binding to purify antigens or antibodies. To purify a specific antigen from a complex mixture of molecules, a monoclonal antibody is attached to an insoluble matrix, such as chromatography beads, and the mixture of molecules is passed over the matrix. The specific antibody binds the antigen of interest; other molecules are washed away. Specific antigen is then eluted by altering the pH, which can usually disrupt antibody-antigen bonds. Antibodies can be purified in the same way on beads coupled to antigen (not shown).
COMPLEMENT FIXATION TEST

(a) Positive test. All available complement is fixed by the antigen–antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen–antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.
EXAMINATION OF COMPLEMENT HAEMOLYTIC ACTIVITY

Functional test of the classical or alternative complement pathway
ELISA (Enzyme-linked Immunosorbent Assay)

immune reaction (Ag-Ab) linked to enzymatic reaction (Enzyme-Substrate)

ELISA types:
- Direct
- Indirect
- "Sandwich"
ELISA
Equipment for ELISA methods
Solid support for ELISA: 96 microwell plastic plate
Immunofluorescence assay

- Immunofluorescence is a technique allowing the visualization of a specific protein or antigen by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC).
- The specific antibodies are labeled with a compound (FITC) that makes them glow an apple-green color when observed microscopically under ultraviolet light.
Detection of Autoantibodies
Fluorescence microscope
Radioimmunoassay (RIA)

1. Add radioactive antigen (tracer) and primary antibody.
2. Add unlabeled antigen.
3. Radioactive antigen displaced by unlabeled antigen.
4. Precipitate Ag-Ab complexes with anti-immunoglobulin (secondary antibody).
5. Radioactivity of supernatant = free antigen (free tracer).
6. Radioactivity of precipitate = bound antigen (bound tracer).
Nephelometry and Turbidimetry

These are the analytical techniques used to measure scattered light.

**Principle of nephelometry** – intensity of light scattered by a suspension is measured at 90 degrees angle.

Intensity of scattered light is a concentration of suspension

**Principle of turbidimetry** – measurement of decrease in light transmitted through a turbid solution is measured.
NEPHELOMETRY

immunoassay system for the quantitative determination of human proteins in serum
disease state diagnosis and monitoring

Determination of basic protein in serum, immunoglobulins IgG, IgA, IgM, IgE, and IgG subclasses, complement components C3 and C4, the acute phase protein (e.g. C-reactive protein, transferrin, alpha-1-macroglobulin, albumin).
A blood test can help in the diagnosis of a type IV allergy. *In vitro* testing with the lymphocyte transformation test (LTT) can detect both dermally and non-dermally (mucosal) sensitizing allergens. It has been used to detect hypersensitivity leading to both local and systemic effects resulting from dental allergies, in particular in the optimized version of LTT known as MELISA (Stjekstal et al, 1994, Muller and Valentine-Thon, 2006). The patient’s lymphocytes are exposed to the allergen to be tested. If they have previously been sensitized to the allergen, they will undergo proliferation, which is measured using incorporation of radioactive nucleotides (tritiated thymidine). Some authors have reported that non-relevant proliferation of lymphocytes could happen in non-sensitized patients (Fisher’s, 2008), leading to some false-positive results.
Mitogen- and Antigen-induced Lymphocyte Proliferation (LTT)

Clinical Significance
Measurement of human lymphocytes' proliferative responses to various stimuli is a fundamental technique used to assess their biological status and functions.

Mitogens, such as plant lectins phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), are able to nonspecifically stimulate lymphocyte proliferation and used to evaluate patient immune responsiveness.
DETERMINATION OF sIgE ANTIBODIES AGAINST ALLERGEN

Basic principle - Enzyme Immunoassay

A variety of the antigen (alllegen) binding methods and detection systems
<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Solid phase matrix</th>
<th>Enzyme-labeled detection antibody</th>
<th>Substrate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Calibration system</th>
<th>Analytical sensitivity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HYTEC-288</td>
<td>Hycor-Agilent</td>
<td>Paper disc</td>
<td>Alkaline phosphatase labeled anti-IgE</td>
<td>4-nitrophenyl phosphate</td>
<td>Total IgE system</td>
<td>0.1 kUa/L</td>
</tr>
<tr>
<td>ImmunoCAP</td>
<td>Phadia</td>
<td>Cellulose sponge</td>
<td>B-galactosidase labeled anti-IgE</td>
<td>4-methyl-umbelliferyl β-D galactoside</td>
<td>Total IgE system</td>
<td>0.1 kUa/L</td>
</tr>
<tr>
<td>Immulite</td>
<td>Siemens</td>
<td>Biotinylated-allergen and avidin particle</td>
<td>Alkaline phosphatase labeled anti-IgE</td>
<td>4-methoxy-4-(3-phosphatephenyl)-spiro-1, 2 dioxetane-3,2′- adamantane</td>
<td>Total IgE system</td>
<td>0.1 kUa/L</td>
</tr>
<tr>
<td>Immuno solid phase Allergen Chip (ISAC)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Phadia</td>
<td>Biochip</td>
<td>B-galactosidase labeled anti-IgE</td>
<td>4-methyl-umbelliferyl β-D galactoside</td>
<td>Positive controls</td>
<td>Semiquantitative</td>
</tr>
</tbody>
</table>
DETERMINATION OF sIgE

Fully automatic system

HYTEC Specific IgE Enzyme Immunoassay
Allergen disc
Allergen-specific IgE from the patient’s serum
Enzyme labelled anti-IgE
Substrate solution
Spectrophotometric measurement
Principle of sIgE detection - chemiluminiscence

Serum and allergens incubate with ligand-coated bead.

Liquid allergens bind to serum antibodies and to the ligand-coated bead.

Antibody conjugate binds to serum IgE.

Substrate initiates chemiluminescent reaction.
BASOPHILS ACTIVATION TESTS
DETECTION OF THE MEDIATORS

<table>
<thead>
<tr>
<th>Eosinophil</th>
<th>Mast cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrogenic and growth factors:</strong></td>
<td><strong>Lipids:</strong></td>
</tr>
<tr>
<td>TGF-β</td>
<td>PGD2, PAF, LT B4, C4, D4, E4, E2 TXA2</td>
</tr>
<tr>
<td>TGF-α</td>
<td>LT C4, D4, E4, PAF, PGE1, PGE2, 15-HETE</td>
</tr>
<tr>
<td>Angiotesin</td>
<td><strong>Cytokines:</strong></td>
</tr>
<tr>
<td>FGF-2</td>
<td>IL-3, -5, -10, -13, -17 TNF α</td>
</tr>
<tr>
<td>HB_EGF</td>
<td><strong>Others:</strong></td>
</tr>
<tr>
<td>NGF</td>
<td>ROI, neuropeptides</td>
</tr>
<tr>
<td>PDGF</td>
<td>MMP</td>
</tr>
<tr>
<td>VEGF</td>
<td><strong>Growth factors:</strong></td>
</tr>
<tr>
<td>SCF</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>GM-CSF</td>
<td><strong>Amines:</strong></td>
</tr>
<tr>
<td><strong>Chemokines:</strong></td>
<td>Histamine</td>
</tr>
<tr>
<td>Eotaxin</td>
<td><strong>Proteoglicans:</strong></td>
</tr>
<tr>
<td>RANTES</td>
<td>Heparine, condroitine</td>
</tr>
<tr>
<td>MCP, -1, -3, -4</td>
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<tr>
<td>IL-8, MIP-1α</td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Proteins:</strong></td>
<td><strong>Chemokines:</strong></td>
</tr>
<tr>
<td>Major Basc Protein; Eosionphil Peroxidase; Eosinophil derived</td>
<td>MIP-1α, IL-8</td>
</tr>
</tbody>
</table>
PROTEIN MICROARRAY
Microarray technology

Experiments

Image scan (laser scanner)

Data extraction (GenePix etc.)

Data computation (Q Analyzer)

Final Result (pg/ml)
The Immunoassay Handbook
Theory and applications of ligand binding, ELISA and related techniques,
D. Wild, 2013
Clinical Laboratory Immunology,
C. R. Mahon and D. Tice, 2006
Clinical Immunology,
Principles and Laboratory Diagnosis,
C. Sheehan, 1996