Histochemistry (304 C)

Third year biochemistry students
Faculty of science
Damietta University

Lecture (5)

Immunohistochemistry

Introduced By

Dr. Rasha F. Zahran

Lecturer of biochemistry

Department of chemistry

Faculty of science

Damietta university

Basic immunology

Definitions

- Immune system = cells, tissues, and molecules that mediate resistance to infections
- Immunology = study of structure and function of the immune system
- Immunity = resistance of a host to pathogens and their toxic effects
- Immune response = collective and coordinated response to the introduction of foreign substances in an individual mediated by the cells and molecules of the immune system

Role of the immune system

- Defense against microbes
- Defense against the growth of tumor cells
  - kills the growth of tumor cells
- Homeostasis
  - destruction of abnormal or dead cells
    (e.g. dead red or white blood cells, antigen-antibody complex)

Immune System

1. Organs
2. Cells
3. Molecules

Immune System: (1) organs

- Tonsils and adenoids
- Thymus
- Lymph nodes
- Spleen
- Peyer's patches
- Appendix
- Lymphatic vessels
- Bone marrow

Immune system: (2) cells

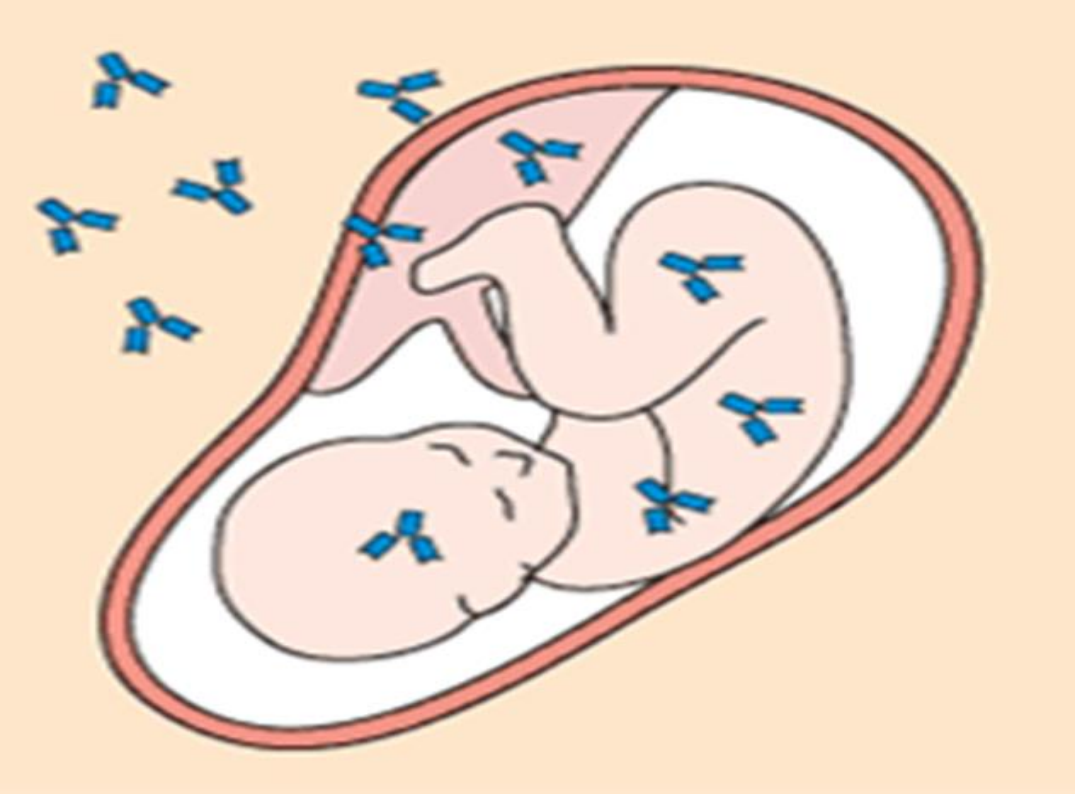
- Lymphocytes
  - T-lymphocytes
  - B-Lymphocytes, plasma cells
  - natural killer lymphocytes
- Monocytes, Macrophage
- Granulocytes
  - neutrophils
  - eosinophils
  - basophils

Immune system: (3) molecules

- **Antibodies:** Antibodies are heavy (~150 kDa) globular plasma proteins. They have sugar chains (glycans) added to conserved amino acid residues.
- **Complement:** The complement system is a group of proteins that move freely through your bloodstream. The proteins work with your immune system. They also play a role in the development of inflammation.
- **Cytokines:** Cytokines serve as messengers between cells and regulate various inflammatory responses, Examples of cytokines include the agents interleukins and the interferon which are involved in regulating the immune system's response to inflammation and infection.

Two types of immunity

1. Innate (non-adaptive)
   - first line of immune response
   - relies on mechanisms that exist before infection
2. Acquired (adaptive)
   - Second line of response (if innate fails)
   - relies on mechanisms that adapt after infection
   - handled by T- and B- lymphocytes
   - one cell determines one antigenic determinant

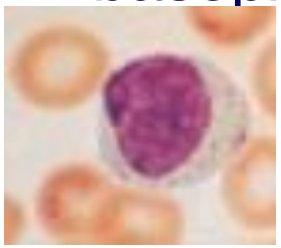


Innate immunity

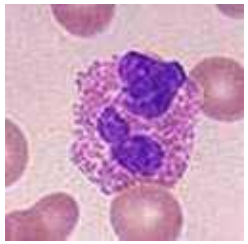
- Based on genetic make-up
- Relies on already formed components
- Rapid response: within minutes of infection
- Not specific
  - same molecules / cells respond to a range of pathogens
- Has no memory
  - same response after repeated exposure
- Does not lead to clonal expansion

Innate immunity: mechanisms

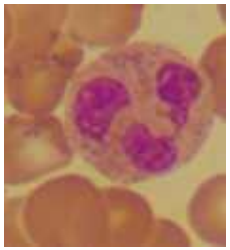
- Mechanical barriers / surface secretion
  - skin, acidic pH in stomach, cilia
- Humoral mechanisms
  - lysozymes, basic proteins, complement, interferons
- Cellular defense mechanisms
  - natural killer cells neutrophils, macrophages,, mast cells, basophils, eosinophils



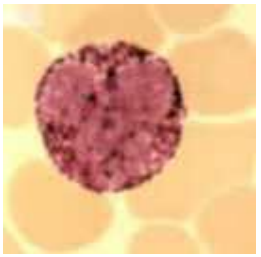
NK Cell



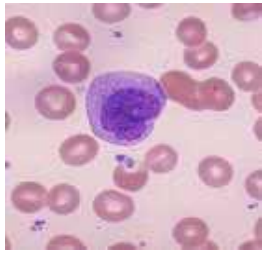
Eosinophils



Neutrophil



Basophils & Mast cells



Monocyte Macrophage

Adaptive immunity: second line of response

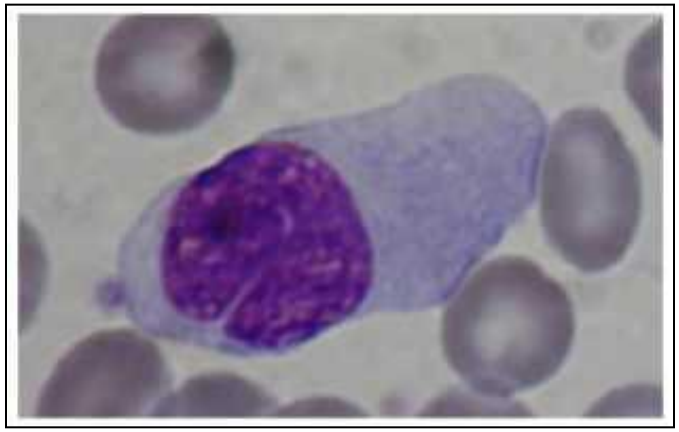
- Based upon resistance acquired during life
- Relies on genetic events and cellular growth
- Responds more slowly, over few days
- Is specific
  - each cell responds to a single epitope on an antigen
- Has anamnestic memory
  - repeated exposure leads to faster, stronger response
- Leads to clonal expansion

Adaptive Immunity: active and passive

	Active Immunity	Passive Immunity
Natural	clinical, sub-clinical infection	via breast milk, placenta
Artificial	Vaccination: Live, killed, purified antigen vaccine	immune serum, immune cells

Adaptive immunity: mechanisms

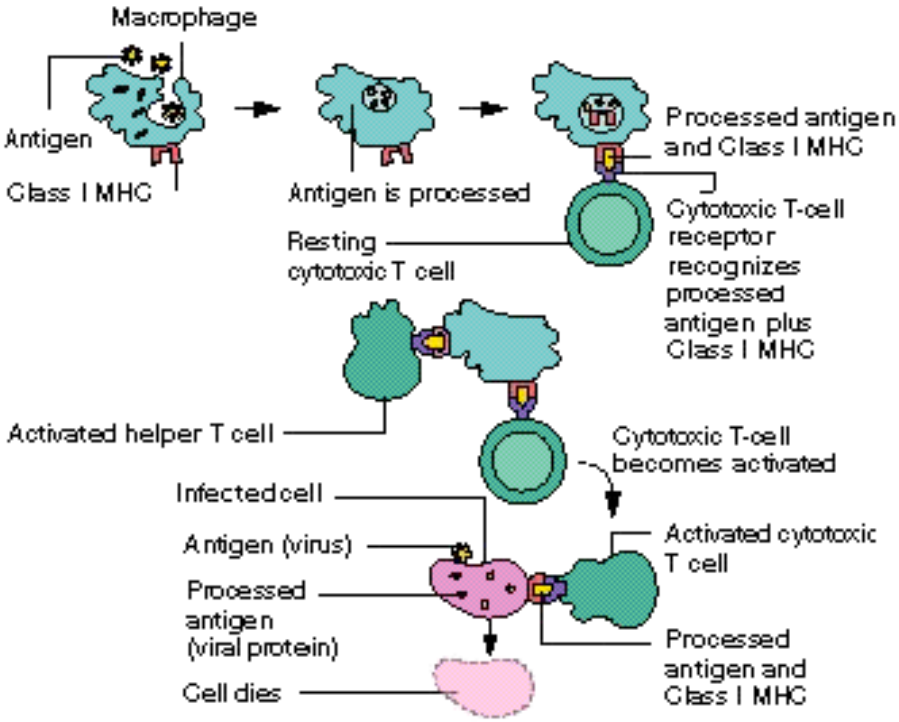
- Cell-mediated immune response (CMIR)
  - T-lymphocytes
  - eliminate intracellular microbes that survive within phagocytes or other infected cells
- Humoral immune response (HIR)
  - B-lymphocytes
  - mediated by antibodies
  - eliminate extra-cellular microbes and their toxins



Plasma cell
(Derived from B-lymphocyte, produces antibodies)

Cell-mediated immune response

1. T-cell
   - recognizes peptide antigen on macrophage in association with major histo-compatibility complex (MHC) class
   - identifies molecules on cell surfaces
   - helps body distinguish self from non-self
2. T-cell goes into effectors cells stage that is able to kill infected cells



T lymphocytes

2 types

- helper T- lymphocytes (CD4+)
  - CD4+ T cells activate phagocytes to kill microbes
- cytolytic T-lymphocyte (CD8+)
  - CD8+ T cells destroy infected cells containing microbes or microbial proteins

Cell mediated immune response

Primary response

- production of specific clones of effector T cells and memory clones
- develops in several days
- does not limit the infection

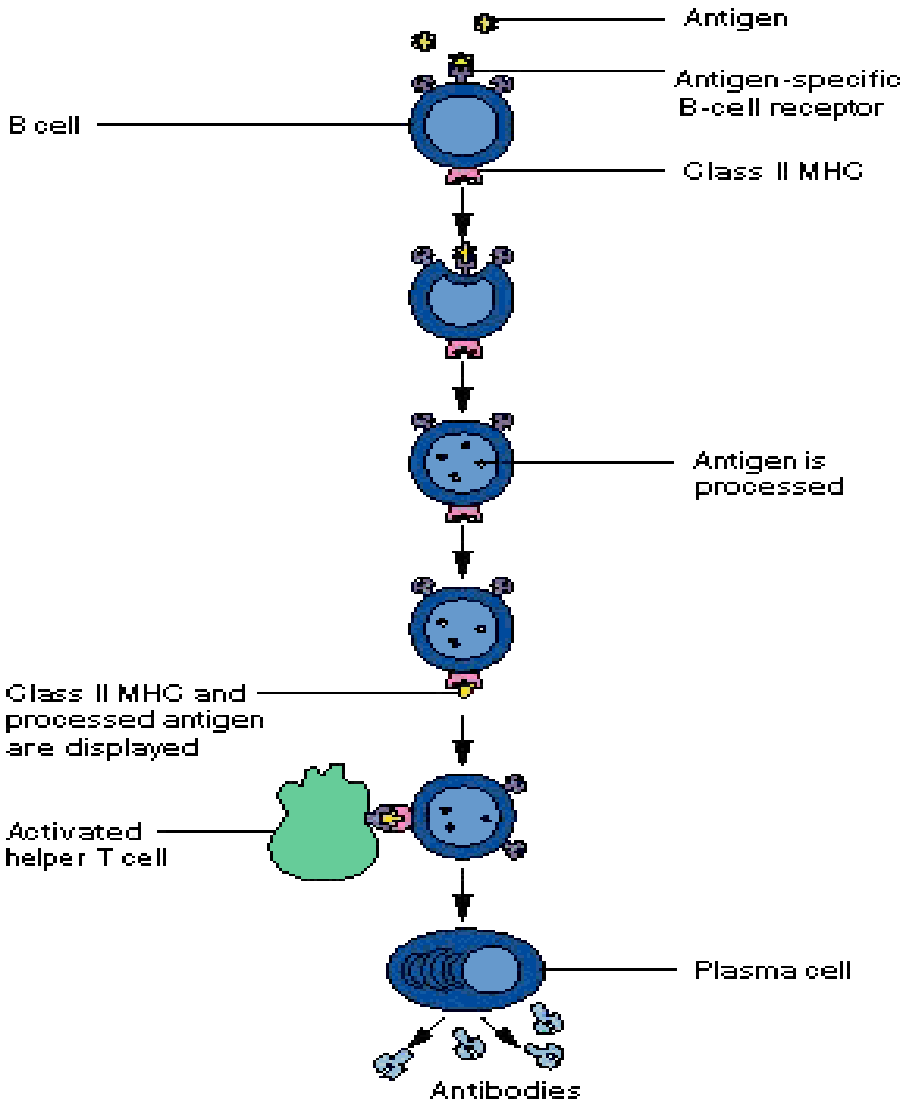
Secondary response

- more pronounced, faster
- more effective at limiting the infection

Example - cytotoxic reactions against intracellular parasites, delayed hypersensitivity (e.g., Tuberculin test) and allograft rejection

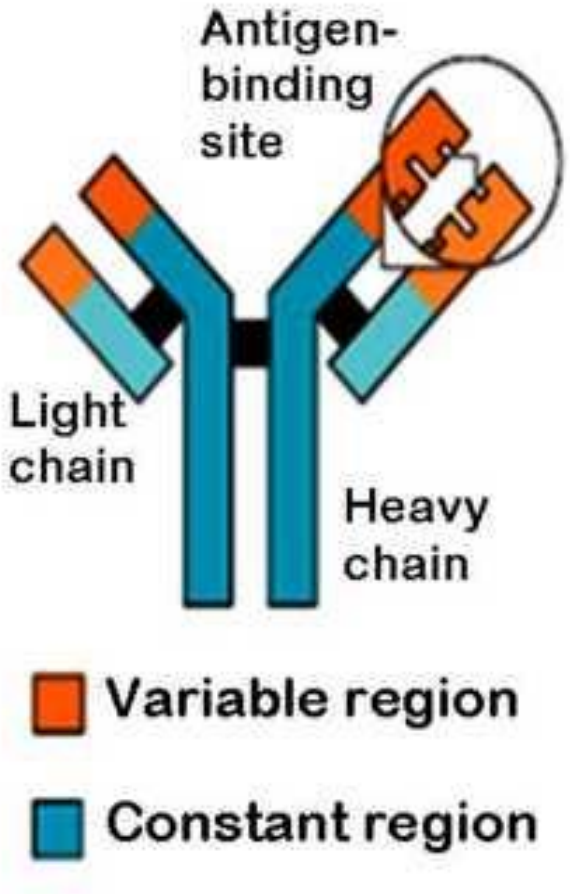
Humoral immune response

1. B lymphocytes recognize specific antigens
   - proliferate and differentiate into antibody-secreting plasma cells
2. Antibodies bind to specific antigens on microbes; destroy microbes via specific mechanisms
3. Some B lymphocytes evolve into the resting state - memory cells



Antibodies (immunoglobulins)

- Belong to the gamma-globulin fraction of serum proteins
- Y-shaped or T-shaped polypeptides
  - 2 identical heavy chains
  - 2 identical light chains
- All immunoglobulins are not antibodies
- Five kinds of antibodies
  - IgG, IgM, IgA, IgD, IgE



IgG

- 70-75% of total immuniglobulin
- Secreted in high quantities in secondary exposures
- Cross the placenta
- Major functions / applications
  - neutralize microbes and toxins
  - opsonize antigens for phagocytosis
  - activate the complement
  - protect the newborn

IgM

- Secreted initially during primary infection
- Cannot cross the placenta
- Major functions / applications
  - secreted first during primary exposure
  - activates the complement
  - used as a marker of recent infection

•Presence in newborn means infection

•Single positive sample in serum or CSF indicates recent or active infection

•Used to detect early phase of infection

IgA

- Monomeric in serum
- Dimeric with secretory component in the lumen of the gastro-intestinal tract and in the respiratory tract
- Major function / application
  - neutralizes microbes and toxins

IgD

- Monomeric
- Major functions / applications
  - present on the surface of B lymphocytes
  - functions as membrane receptor
  - role unclear
    - has a role in antigen stimulated lymphocyte differentiation

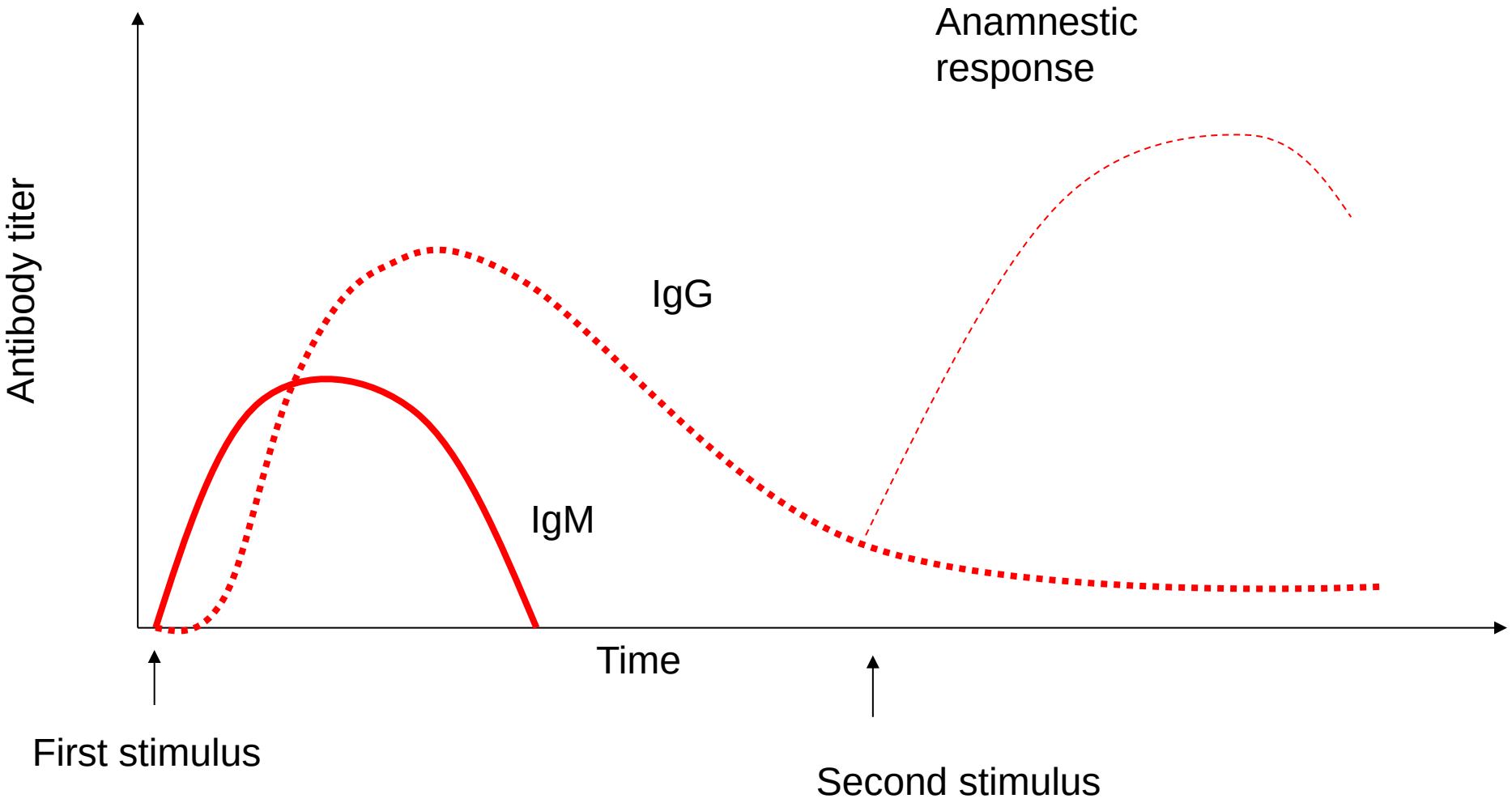
IgE

- Mediates type I hypersensitivity
- Monomeric
- Major functions / applications
  - associated with anaphylaxis
  - plays a role in immunity to helminthic parasites

Sequential IgM-IgG humoral response

- IgM
  - produced as a first response to many antigens
  - levels remain high transiently
- IgG
  - produced after IgM
  - higher levels persist in small amounts throughout life
  - produced in large amounts during secondary response
    - persistence of antigen sensitive ‘memory cells’ after primary response

IgM – IgG sequential response



Failure of immune response

- Immune response helps individuals defend against
  - microbes
  - some cancers
- Immune response can fail
  - hypersensitivity reactions
  - immunodeficiency

Hypersensitivity reactions

- Cause cell damage through excessive immune response to antigens
- Hypersensitivity
  - overreaction to infectious agents
- Allergy
  - overreaction to environmental substances
- Autoimmunity
  - overreaction to self

Immunodeficiency

- Congenital (primary) immunodeficiency
  - genetic abnormality
    - defect in lymphocyte maturation
- Acquired (secondary) immunodeficiency
  - results from infections, nutritional deficiencies or treatments
    - AIDS, chronic leukemia

Summary (1)

- Innate immunity
  - relies on mechanisms already existing before microbe infects host
  - is the first line of defense
  - has no memory for subsequent exposure
  - relies on non specific mechanisms

Summary (2)

- Adaptive immunity
  - develops following entry of microbe into the host
  - comes into action after innate immunity fails to get rid of microbe
  - has memory to deal with subsequent exposure
  - happens through specific cells
    - T cells (cell mediated)
    - B cells (antibody mediated)

Summary (3)

- Primary immune response
  - short lasting
  - smaller in magnitude
- Secondary immune response
  - longer in duration
  - larger in magnitude
    - develop ‘memory cells’ following primary response
- Failure of immune response can result in:
  - hypersensitivity
  - immunodeficiency

Immunohistochemistry (IHC)

Staining of cells and sections with antibodies has transformed histology, since it allows the identification and localization of individual molecules. A molecule that binds to and is recognized by an antibody is called an antigen, and in the context of histology, such antigens are often referred to as markers, since as ways of recognizing a they act particular cell.

Immunohistochemistry (IHC):

refers to the process of selectively imaging antigens (e.g. proteins) in cells of a tissue section by using the principle of antibodies binding specifically to antigens in biological tissues. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein .

The technique is particularly valuable for **distinguishing different cell types in the diagnosis of cancer.** For example, different classes of lymphocyte appear
virtually identical in size and shape (morphology), but they can be distinguished according
to their surface markers - all T lymphocytes express CD3, and the two major
subpopulations of helper T cells and cytotoxic T cells express CD4 and CD8 respectively.
So a T cell lymphoma can be tracked in different tissues using these markers. The CD
system identifies surface molecules on different cells and includes more than 250 different
proteins. .

Immunohistochemical staining is widely used in the **diagnosis of abnormal cells** such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as **proliferation or cell death (apoptosis).**
Immunohistochemistry is also widely used in basic research to **understand the distribution and localization of biomarkers** and differentially expressed proteins in different parts of a biological tissue.

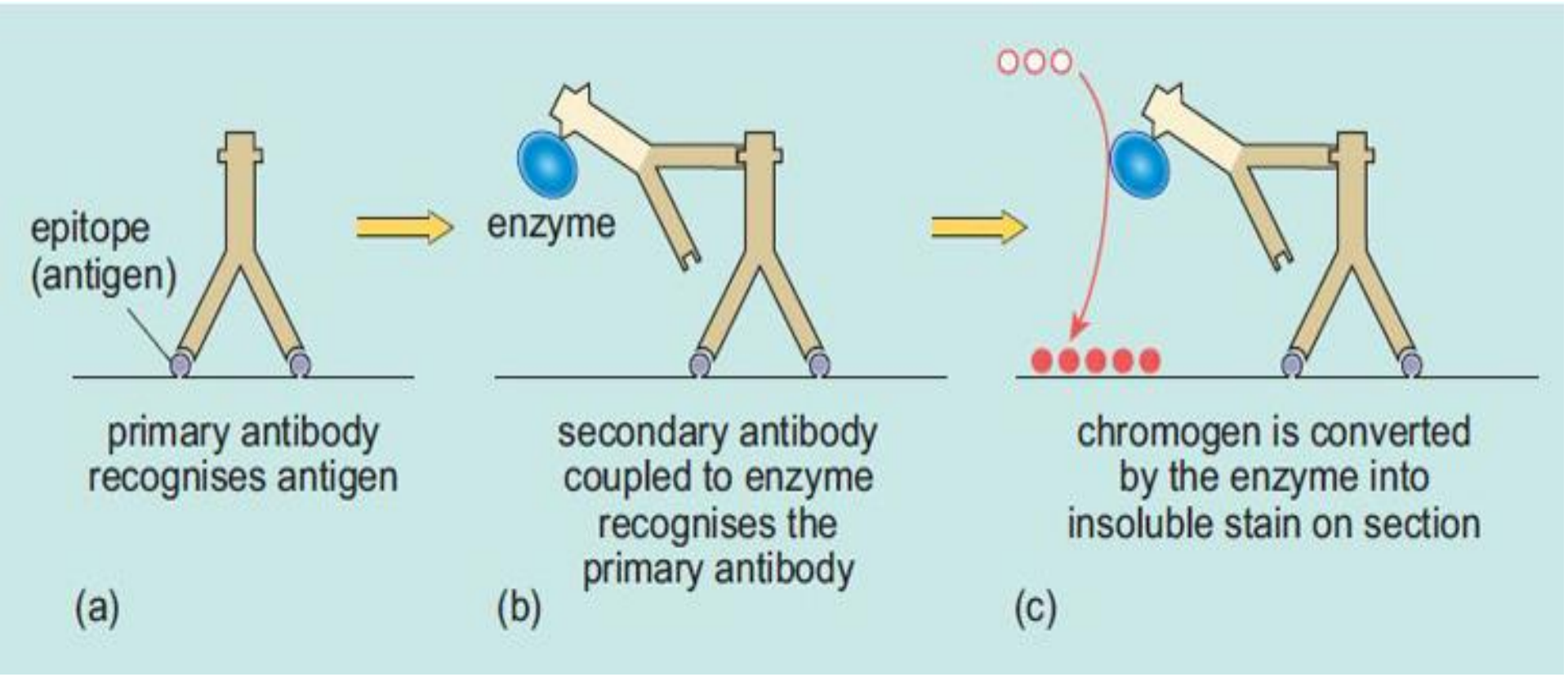
Another use for immunohistochemistry is in **guiding treatment**. For example, the drug
tamoxifen is used to treat breast cancer; many breast cancers require estrogen to divide
and tamoxifen binds to the oestrogen-receptor on the cells, blocking this proliferative
effect. However, this only applies to some cancers; some do not have an oestrogen
receptor and are therefore not susceptible to tamoxifen therapy. A histologist can use
immunohistochemistry to identify whether a breast cancer removed by surgery expresses the oestrogen receptor (which in this context is a cell surface marker identified by an
antibody). The clinician wants to treat the patient so as to prevent any secondary tumours
from growing and with this information they can decide whether or not tamoxifen therapy
is appropriate

Immunohistochemistry protocol

A standard immunohistochemistry protocol involves treatment of the section with an antibody that recognizes the marker. This is referred to as the primary antibody. Then the primary antibody is recognized by a secondary antibody that is linked to an enzyme, or several copies of the enzymes - such reagents are sometimes called conjugates,

Finally,

the section is treated with a chromogen, a reagent that is acted upon by the enzyme, to deposit an insoluble coloured compound onto the cell, where the original primary antibody had bound. Extensive washing is required between each of the stages to prevent non-specific binding and so that the previous reagent does not neutralize the next reagent before it has a chance to bind to the cell. The system is outlined in Figure 1.



Principles of IHC. Indirect immunolabelling: unlabelled primary antibodies bind to
to the antigen on the section. Labelled secondary antibodies bind to the primary
antibodies. The secondary antibodies have an enzyme attached which acts on a
substrate to deposit a coloured reagent where the antigen is located.

Non-specific binding : can either be due to the antibody binding (weakly) to the cells when no antigen is present, or may be due to the antibody cross-reacting, i.e. binding specifically and strongly to another antigen that has a similar structure to the target antigen. Generally one aims to avoid using antibodies that cross-react, if at all possible. There are also a number of ways of reducing weak non-specific binding, .

To reduce background staining in IHC and other immunostaining methods samples are **incubated with**
a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA, or gelatin. Methods to eliminate background staining include **dilution of the primary or secondary antibodies, changing the time or temperature of incubation**, and using a different detection system or different primary antibody.

Quality control should as a minimum include a tissue known to express the antigen as a positive control, and negative controls of tissue known not to express the antigen, as well as the test tissue probed in the same way with omission of the primary antibody (or better, absorption of the primary antibody).

What considerations are important in selecting a primary antibody? Give three points.

Answer

It should be specific for the target antigen. It needs to be able to react with tissues that have been fixed (or non-fixed tissue can be used). It has to be recognizable by the secondary antibody.

Most primary antibodies will be IgG, because IgG antibodies typically have a high affinity for their antigen, and are therefore more likely to be highly specific for their target antigen.

Give three considerations in selecting a secondary antibody for use in a histology laboratory.

Answer

It is advantageous if the secondary can recognize a wide range of primary antibodies since it is more versatile. The level of enzyme conjugated to the antibody affects the sensitivity of the test. This reagent will be used often, so cost is also important.

Antibodies are expensive reagents, so everyone aims to use the minimum amount, by localizing the drop of antibody that is needed to cover the tissue section. It is the concentration, rather than the volume that is important and volumes of as little as 50µl of diluted antibody can be used.

A variety of enzymes may be conjugated to the secondary antibody, but peroxidase is most commonly used. Peroxidase uses H_2O_2 as one of its substrates, so the chromogen mixture must contain an optimal amount of the substrate. The enzyme itself can be damaged by H_2O_2 , if too much is present.

Apart from the substrates, what other to considerations are important for the enzyme work?

Answer: Enzymes work at a particular optimum pH and temperature, so the chromogen solution must be buffered at this pH, and the reaction should take place for the amount of time, at the right correct temperature