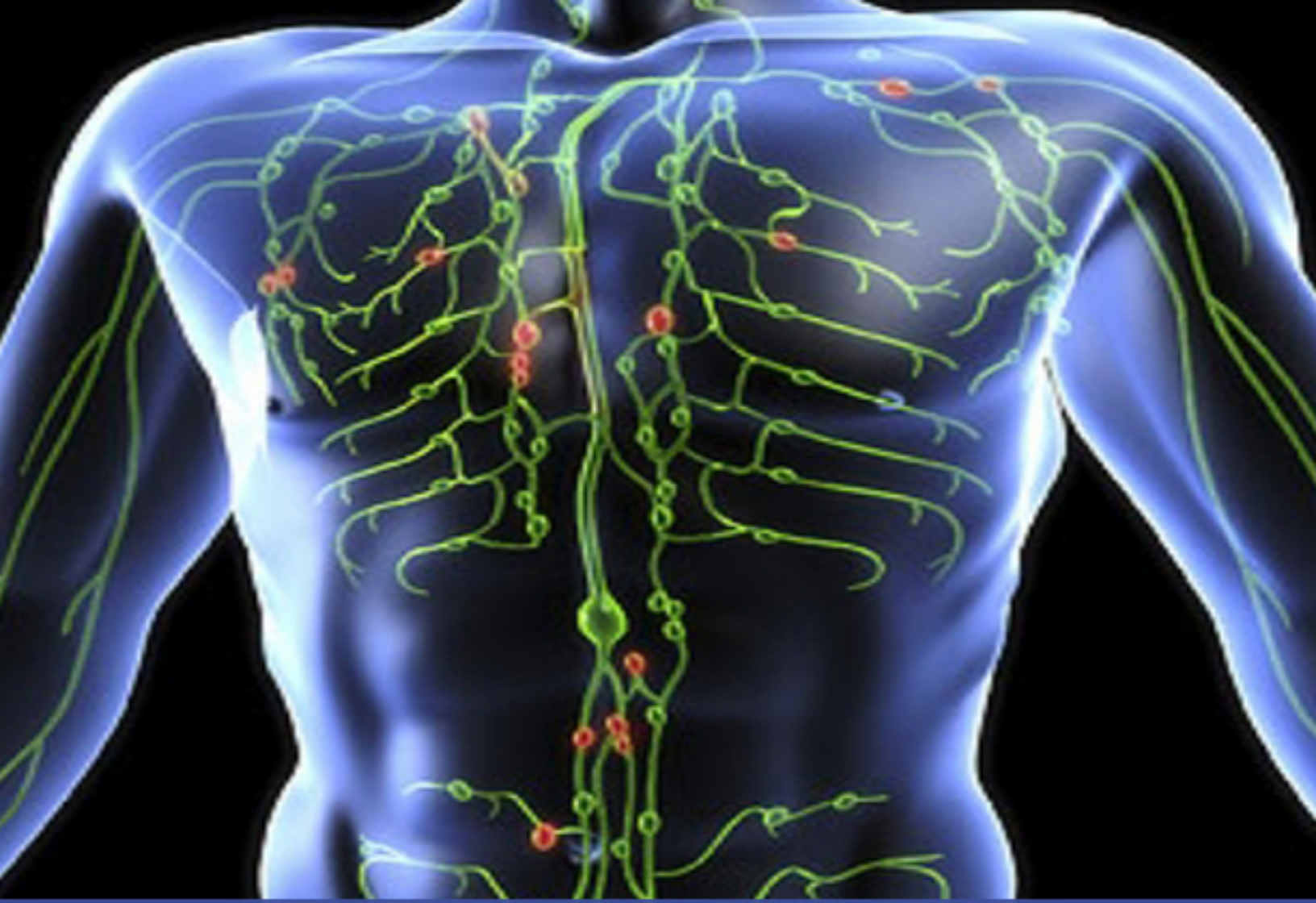

Lymphatic System and Procedures

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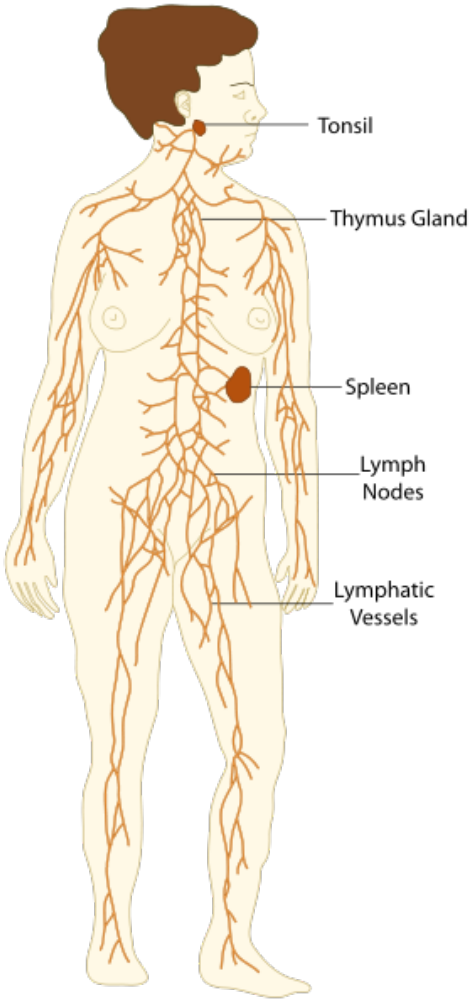
Chapter 1

Lymphatic System

Lymphatic System



An image displaying the lymphatic system.

Latin *systema lymphoideum*

The **lymphatic system** is the part of the immune system comprising a network of conduits called lymphatic vessels that carry a clear fluid called lymph (from Latin *lympha* "water") unidirectionally toward the heart. Lymphoid tissue is found in many organs, particularly the lymph nodes, and in the lymphoid follicles associated with the digestive system such as the tonsils. The system also includes all the structures dedicated to the circulation and production of lymphocytes, which includes the spleen, thymus, bone marrow and the lymphoid tissue associated with the digestive system. The lymphatic system as we know it today was first described independently by Olaus Rudbeck and Thomas Bartholin.

The blood does not directly come in contact with the parenchymal cells and tissues in the body, but constituents of the blood first exit the microvascular exchange blood vessels to become interstitial fluid, which comes into contact with the parenchymal cells of the body. Lymph is the fluid that is formed when interstitial fluid enters the initial lymphatic vessels of the lymphatic system. The lymph is then moved along the lymphatic vessel network by either intrinsic contractions of the lymphatic vessels or by extrinsic compression of the lymphatic vessels via external tissue forces (e.g. the contractions of skeletal muscles).

Function

The lymphatic system has multiple interrelated functions:

- it is responsible for the removal of interstitial fluid from tissues
- it absorbs and transports fatty acids and fats as chyle to the circulatory system
- it transports immune cells to and from the lymph nodes in to the bone
- The lymph transports antigen-presenting cells (APCs), such as dendritic cells, to the lymph nodes where an immune response is stimulated.

Lymphatic tissue is a specilized connective tissue - reticular connective, that contains large quantities of lymphocytes.

Clinical significance

The study of lymphatic drainage of various organs is important in diagnosis, prognosis, and treatment of cancer. The lymphatic system, because of its physical proximity to many tissues of the body, is responsible for carrying cancerous cells between the various parts of the body in a process called metastasis. The intervening lymph nodes can trap the cancer cells. If they are not successful in destroying the cancer cells the nodes may become sites of secondary tumors.

Organization

The lymphatic system can be broadly divided into the conducting system and the lymphoid tissue.

- The conducting system carries the lymph and consists of tubular vessels that include the lymph capillaries, the lymph vessels, and the right and left thoracic ducts.

- The lymphoid tissue is primarily involved in immune responses and consists of lymphocytes and other white blood cells enmeshed in connective tissue through which the lymph passes. Regions of the lymphoid tissue that are densely packed with lymphocytes are known as *lymphoid follicles*. Lymphoid tissue can either be structurally well organized as lymph nodes or may consist of loosely organized lymphoid follicles known as the mucosa-associated lymphoid tissue (MALT)

Lymphoid tissue

Lymphoid tissue associated with the lymphatic system is concerned with immune functions in defending the body against the infections and spread of tumors. It consists of connective tissue with various types of white blood cells enmeshed in it, most numerous being the lymphocytes.

The lymphoid tissue may be primary, secondary, or tertiary depending upon the stage of lymphocyte development and maturation it is involved in. (The tertiary lymphoid tissue typically contains far fewer lymphocytes, and assumes an immune role only when challenged with antigens that result in inflammation. It achieves this by importing the lymphocytes from blood and lymph.)

Primary lymphoid organs

The **central or primary lymphoid organs** generate lymphocytes from immature progenitor cells.

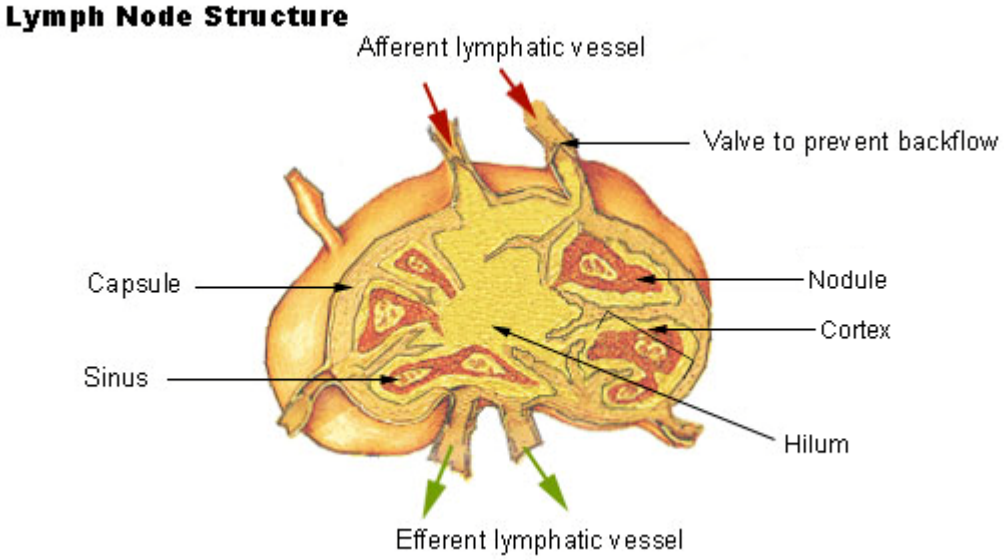
The thymus and the bone marrow constitute the primary lymphoid tissues involved in the production and early selection of lymphocytes.

Secondary lymphoid organs

Secondary or peripheral lymphoid organs maintain mature naive lymphocytes and initiate an adaptive immune response. The peripheral lymphoid organs are the sites of lymphocyte activation by antigen. Activation leads to clonal expansion and affinity maturation. Mature lymphocytes recirculate between the blood and the peripheral lymphoid organs until they encounter their specific antigen.

Secondary lymphoid tissue provides the environment for the foreign or altered native molecules (antigens) to interact with the lymphocytes. It is exemplified by the lymph nodes, and the lymphoid follicles in tonsils, Peyer's patches, spleen, adenoids, skin, etc. that are associated with the mucosa-associated lymphoid tissue (MALT).

Lymph nodes



A lymph node showing afferent and efferent lymphatic vessels

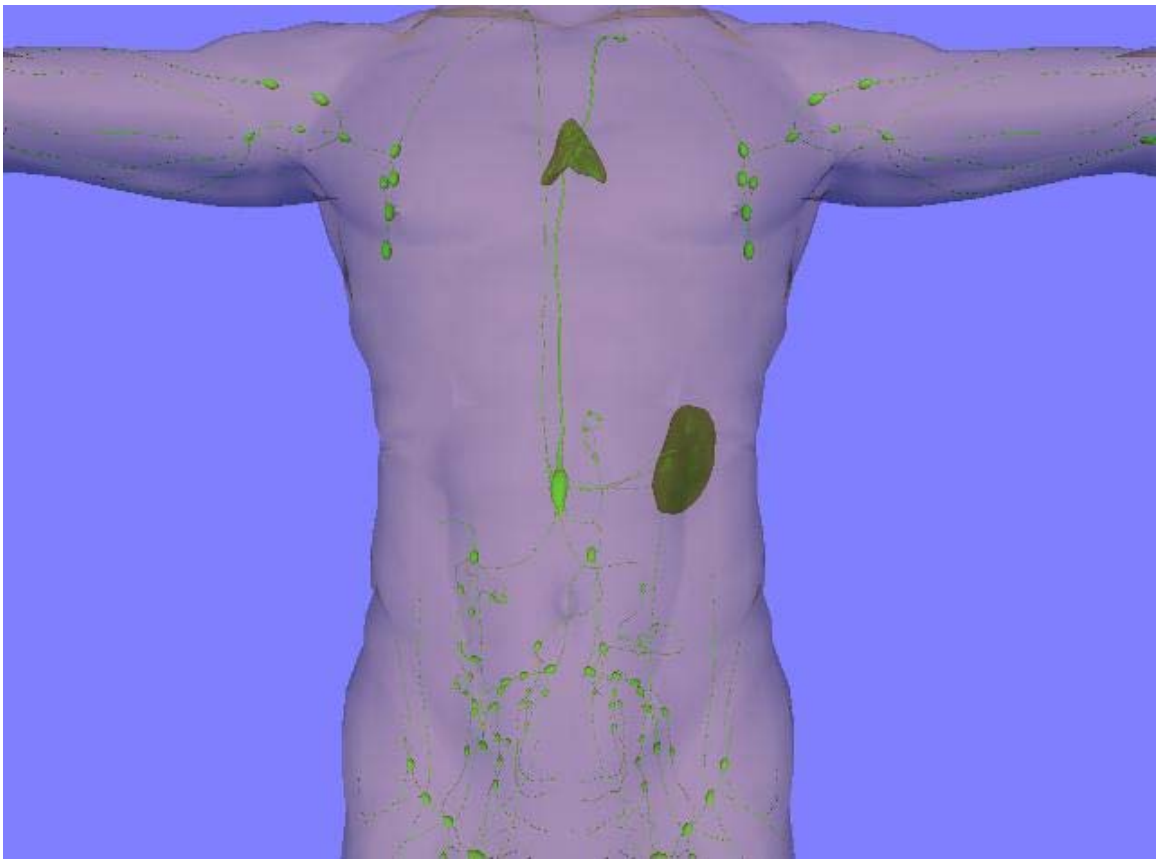
A lymph node is an organized collection of lymphoid tissue, through which the lymph passes on its way to returning to the blood. Lymph nodes are located at intervals along the lymphatic system. Several afferent lymph vessels bring in lymph, which percolates through the substance of the lymph node, and is drained out by an efferent lymph vessel.

The substance of a lymph node consists of lymphoid follicles in the outer portion called the "cortex", which contains the lymphoid follicles, and an inner portion called "medulla", which is surrounded by the cortex on all sides except for a portion known as the "hilum". The hilum presents as a depression on the surface of the lymph node, which makes the otherwise spherical or ovoid lymph node bean-shaped. The efferent lymph vessel directly emerges from the lymph node here. The arteries and veins supplying the lymph node with blood enter and exit through the hilum.

Lymph follicles are a dense collection of lymphocytes, the number, size and configuration of which change in accordance with the functional state of the lymph node. For example, the follicles expand significantly upon encountering a foreign antigen. The selection of B cells occurs in the germinal center of the lymph nodes.

Lymph nodes are particularly numerous in the mediastinum in the chest, neck, pelvis, axilla (armpit), inguinal (groin) region, and in association with the blood vessels of the intestines.

Lymphatics



Lymphatic System

Tubular vessels transport back lymph to the blood ultimately replacing the volume lost from the blood during the formation of the interstitial fluid. These channels are the lymphatic channels or simply called *lymphatics*.

Function of the fatty acid transport system

Lymph vessels called lacteals are present in the lining of the gastrointestinal tract, predominantly in the small intestine. While most other nutrients absorbed by the small intestine are passed on to the portal venous system to drain via the portal vein into the liver for processing, fats (lipids) are passed on to the lymphatic system to be transported to the blood circulation via the thoracic duct. (There are exceptions, for example Medium chain triglycerides (MCTs) are fatty acid esters of glycerol that passively diffuse from the GI tract to the portal system.) The enriched lymph originating in the lymphatics of the small intestine is called chyle. As the blood circulates, fluid leaks out into the body tissues. This fluid is important because it carries food to the cells and waste back to the bloodstream. The nutrients that are released to the circulatory system are processed by the liver, having passed through the systemic circulation. The lymph system is a one-way system, transporting interstitial fluid back to blood.

Diseases of the lymphatic system

Lymphedema is the swelling caused by the accumulation of lymph fluid, which may occur if the lymphatic system is damaged or has malformations. It usually affects the limbs, though face, neck and abdomen may also be affected.

Some common causes of swollen lymph nodes include infections, infectious mononucleosis, and cancer, e.g. Hodgkin's and non-Hodgkin's lymphoma, and metastasis of cancerous cells via the lymphatic system. In elephantiasis, infection of the lymphatic vessels cause a thickening of the skin and enlargement of underlying tissues, especially in the legs and genitals. It is most commonly caused by a parasitic disease known as lymphatic filariasis. Lymphangiosarcoma is a malignant soft tissue tumor (soft tissue sarcoma), whereas lymphangioma is a benign tumor occurring frequently in association with Turner syndrome. Lymphangioleiomyomatosis is a benign tumor of the smooth muscles of the lymphatics that occurs in the lungs.

Development of lymphatic tissue

Lymphatic tissues begin to develop by the end of the fifth week of embryonic development. Lymphatic vessels develop from lymph sacs that arise from developing veins, which are derived from mesoderm.

The first lymph sacs to appear are the paired jugular lymph sacs at the junction of the internal jugular and subclavian veins. From the jugular lymph sacs, lymphatic capillary plexuses spread to the thorax, upper limbs, neck and head. Some of the plexuses enlarge and form lymphatic vessels in their respective regions. Each jugular lymph sac retains at least one connection with its jugular vein, the left one developing into the superior portion of the thoracic duct.

The next lymph sac to appear is the unpaired retroperitoneal lymph sac at the root of the mesentery of the intestine. It develops from the primitive vena cava and mesonephric veins. Capillary plexuses and lymphatic vessels spread from the retroperitoneal lymph sac to the abdominal viscera and diaphragm. The sac establishes connections with the cisterna chyli but loses its connections with neighboring veins.

The last of the lymph sacs, the paired posterior lymph sacs, develop from the iliac veins. The posterior lymph sacs produce capillary plexuses and lymphatic vessels of the abdominal wall, pelvic region, and lower limbs. The posterior lymph sacs join the cisterna chyli and lose their connections with adjacent veins.

With the exception of the anterior part of the sac from which the cisterna chyli develops, all lymph sacs become invaded by mesenchymal cells and are converted into groups of lymph nodes.

The spleen develops from mesenchymal cells between layers of the dorsal mesentery of the stomach. The thymus arises as an outgrowth of the third pharyngeal pouch.

History

Hippocrates was one of the first persons to mention the lymphatic system in 5th century BC. In his work "On Joints," he briefly mentioned the lymph nodes in one sentence. Rufus of Ephesus, a Roman physician, identified the axillary, inguinal and mesenteric lymph nodes as well as the thymus during the 1st to 2nd century AD. The first mention of lymphatic vessels was in 3rd century BC by Herophilus, a Greek anatomist living in Alexandria, who incorrectly concluded that the "absorptive veins of the lymphatics", by which he meant the lacteals (lymph vessels of the intestines), drained into the hepatic portal veins, and thus into the liver. Findings of Ruphus and Herophilus findings were further propagated by the Greek physician Galen, who described the lacteals and mesenteric lymph nodes which he observed in his dissection of apes and pigs in the 2nd century AD.

Until the 17th century, ideas of Galen were most prevalent. Accordingly, it was believed that the blood was produced by the liver from chyle contaminated with ailments by the intestine and stomach, to which various spirits were added by other organs, and that this blood was consumed by all the organs of the body. This theory required that the blood be consumed and produced many times over. His ideas had remained unchallenged until the 17th century, and even then were defended by some physicians.

Olaus Rudbeck in 1696

In the mid 16th century Gabriel Fallopius (discoverer of the Fallopian Tubes) described what are now known as the lacteals as "coursing over the intestines full of yellow matter." In about 1563 Bartolomeo Eustachi, a professor of anatomy, described the thoracic duct in horses as *vena alba thoracis.* The next breakthrough came when in 1622 a physician, Gasparo Aselli, identified lymphatic vessels of the intestines in dogs and termed them *venae alba et lacteae,* which is now known as simply the lacteals. The lacteals were termed the fourth kind of vessels (the other three being the artery, vein and nerve, which was then believed to be a type of vessel), and disproved Galen's assertion that chyle was carried by the veins. But, he still believed that the lacteals carried the chyle to the liver (as taught by Galen). He also identified the thoracic duct but failed to notice its connection with the lacteals. This connection was established by Jean Pecquet in 1651, who found a white fluid mixing with blood in a dog's heart. He suspected that fluid to be chyle as its flow increased when abdominal pressure was applied. He traced this fluid to the thoracic duct, which he then followed to a chyle-filled sac he called the *chyli receptaculum,* which is now known as the cisternae chyli; further investigations led him to find that lacteals' contents enter the venous system via the thoracic duct. Thus, it was proven convincingly that the lacteals did not terminate in the liver, thus disproving Galen's second idea: that the chyle flowed to the liver. Johann Veslingius drew the earliest sketches of the lacteals in humans in 1647.

Thomas Bartholin

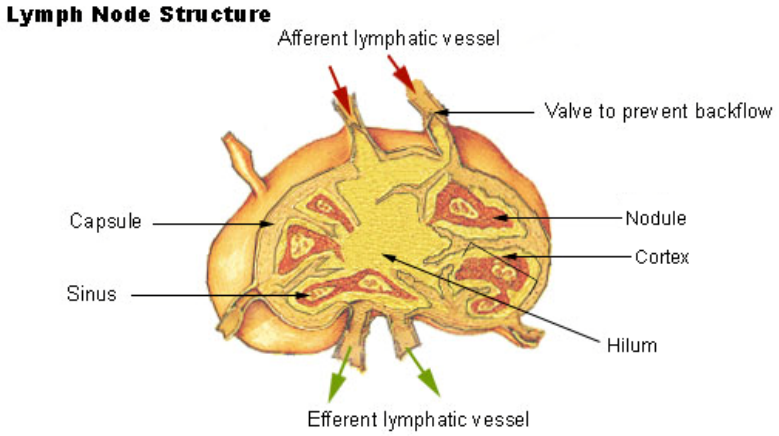
The idea that blood recirculates through the body rather than being produced anew by the liver and the heart was first accepted as a result of works of William Harvey—a work he published in 1628. In 1652, Olaus Rudbeck (1630–1702), a Swede, discovered certain

transparent vessels in the liver that contained clear fluid (and not white), and thus named them *hepatico-aqueous vessels*. He also learned that they emptied into the thoracic duct, and that they had valves. He announced his findings in the court of Queen Christina of Sweden, but did not publish his findings for a year, and in the interim similar findings were published by Thomas Bartholin, who additionally published that such vessels are present everywhere in the body, and not just the liver. He is also the one to have named them "lymphatic vessels". This had resulted in a bitter dispute between one of Bartholin's pupils, Martin Bogdan, and Rudbeck, whom he accused of plagiarism.

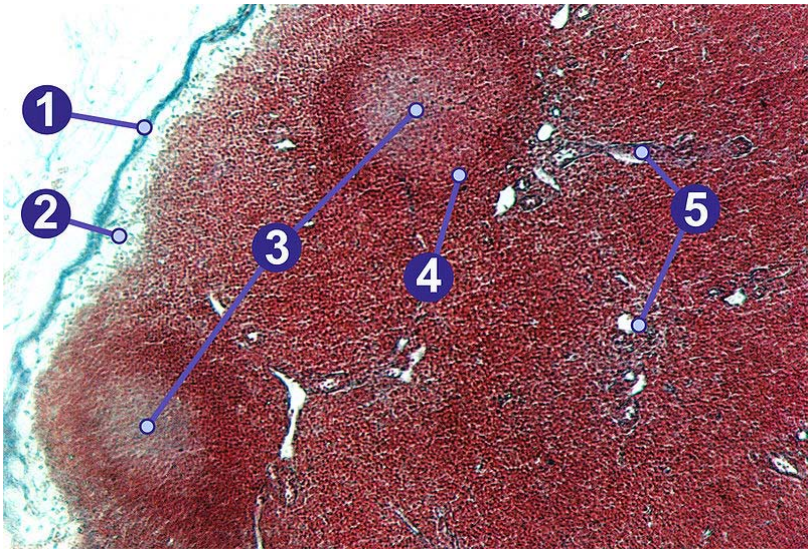
Chapter 2

Lymph Node

Lymph node



A lymph node showing afferent and efferent lymphatic vessels



Lymph node, showing (1) capsule, (2) subcapsular sinus, (3) germinal centers, (4) lymphoid nodule, (5) trabeculae.

Latin *nodus lymphaticus*

A **lymph node** is a small ball-shaped organ of the immune system, distributed widely throughout the body including the armpit and stomach/gut and linked by lymphatic vessels. Lymph nodes are garrisons of B, T, and other immune cells. Lymph nodes are found all through the body, and act as filters or traps for foreign particles. They are important in the proper functioning of the immune system.

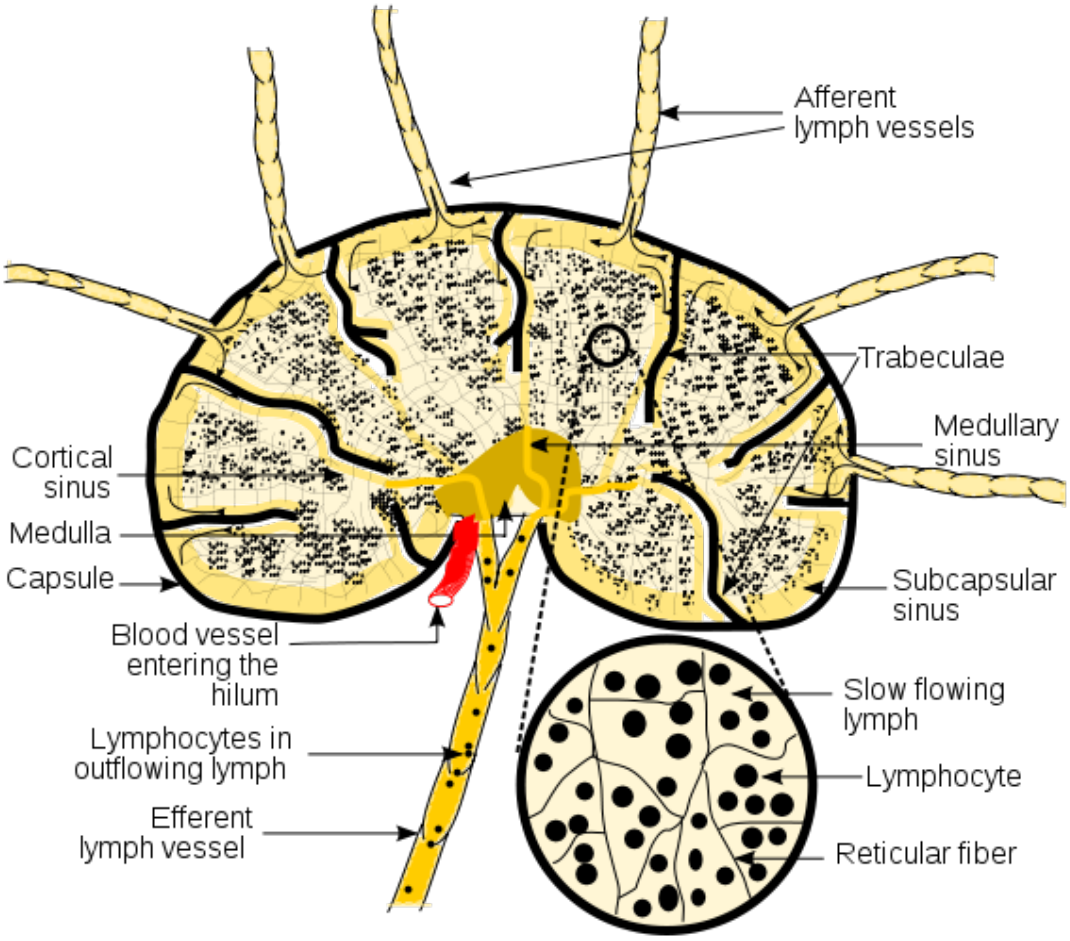
Lymph nodes also have clinical significance. They become inflamed or enlarged in various conditions, which may range from trivial, such as a throat infection, to life-threatening such as cancers. In the latter, the condition of lymph nodes is so significant that it is used for cancer staging, which decides the treatment to be employed, and for determining the prognosis.

Lymph nodes can also be diagnosed by biopsy whenever they are inflamed. Certain diseases affect lymph nodes with characteristic consistency and location.

Function

Pathogens, or germs, can set up infections anywhere in the body. However, lymphocytes, a type of white blood cell, will meet the antigens, or proteins, in the peripheral lymphoid organs, which includes lymph nodes. The antigens are displayed by specialized cells in the lymph nodes. Naive lymphocytes (meaning the cells have not encountered an antigen yet) enter the node from the bloodstream, through specialized capillary venules. After the lymphocytes specialize they will exit the lymph node through the efferent lymphatic vessel with the rest of the lymph. The lymphocytes continuously recirculate the peripheral lymphoid organs and the state of the lymph nodes depends on infection. During an infection, the lymph nodes can expand due to intense B-cell proliferation in the germinal centers, a condition commonly referred to as "swollen glands".

Structure



Schematic diagram of lymph node showing the flow of lymph through the lymph sinuses. Note: Outflowing lymph has more lymphocytes

The lymph node is surrounded by a fibrous capsule, and inside the lymph node the fibrous capsule extends to form trabeculae. The substance of the lymph node is divided into the outer cortex and the inner medulla surrounded by the former all around except for at the hilum, where the medulla comes in direct contact with the surface.

Thin reticular fibers, elastin and reticular fibers form a supporting meshwork called *reticular network* (RN) inside the node, within which the white blood cells (WBCs), the most prominent ones being lymphocytes, are tightly packed as follicles in the cortex. Elsewhere, there are only occasional WBCs. The RN provides not just the structural support, but also will provide surface for adhesion of the dendritic cells, macrophages and lymphocytes. It allows for exchange of material with blood through the high endothelial venules and provides the growth and regulatory factors necessary for activation and maturation of immune cells.

The number and composition of follicles can change especially when challenged by an antigen, when they develop a germinal center.

A lymph sinus is a channel within the lymph node lined by the endothelial cells along with fibroblastic reticular cells and allows for smooth flow of lymph through them. Thus, subcapsular sinus is a sinus immediately deep to the capsule, and its endothelium is continuous with that of the afferent lymph vessel. It is also continuous with similar sinuses flanking the trabeculae and within the cortex (cortical sinuses). The cortical sinuses and that flanking the trabeculae drain into the *medullary sinuses*, from where the lymph flows into the efferent lymph vessel.

Multiple afferent lymph vessels that branch and network extensively within the capsule bring lymph into the lymph node. This lymph enters the subcapsular sinus. The innermost lining of the afferent lymph vessels is continuous with the cells lining the lymph sinuses. The lymph gets slowly filtered through the substance of the lymph node and ultimately reaches the medulla. In its course it encounters the lymphocytes and may lead to their activation as a part of adaptive immune response.

The concave side of the lymph node is called the hilum. The efferent attaches to the hilum by a relatively dense reticulum present there, and carries the lymph out of the lymph node.

Cortex

In the cortex, the subcapsular sinus drains to *trabecular sinuses*, and then the lymph flows into the "medullary sinuses".

The outer cortex consists mainly of the B cells arranged as follicles, which may develop a germinal center when challenged with an antigen, and the deeper cortex mainly consisting of the T cells. There is a zone known as the subcortical zone where T-cells (or cells that are mainly red) mainly interact with dendritic cells, and where the reticular network is dense.

Medulla

There are two named structures in the medulla:

- The *medullary cords* are cords of lymphatic tissue, and include plasma cells, macrophages, and B cells
- The *medullary sinuses* (or *sinusoids*) are vessel-like spaces separating the medullary cords. The Lymph flows into the medullary sinuses from cortical sinuses, and into efferent lymphatic vessels. Medullary sinuses contain histiocytes (immobile macrophages) and reticular cells.

Shape and size

Human lymph nodes are bean-shaped and range in size from a few millimeters to about 1–2 cm in their normal state. They may become enlarged due to a tumor or infection, or inflamed due to leukemia. Lymphocytes, also known as white blood cells, are located within honeycomb structures of the lymph nodes. Lymph nodes are enlarged when the body is infected, primarily because there is an elevated rate of trafficking of lymphocytes into the node from the blood, exceeding the rate of outflow from the node, and secondarily as a result of the activation and proliferation of antigen-specific T and B cells (clonal expansion). In some cases, they may feel enlarged because of a previous infection; although one may be healthy, one may still feel them residually enlarged.

Lymphatic circulation

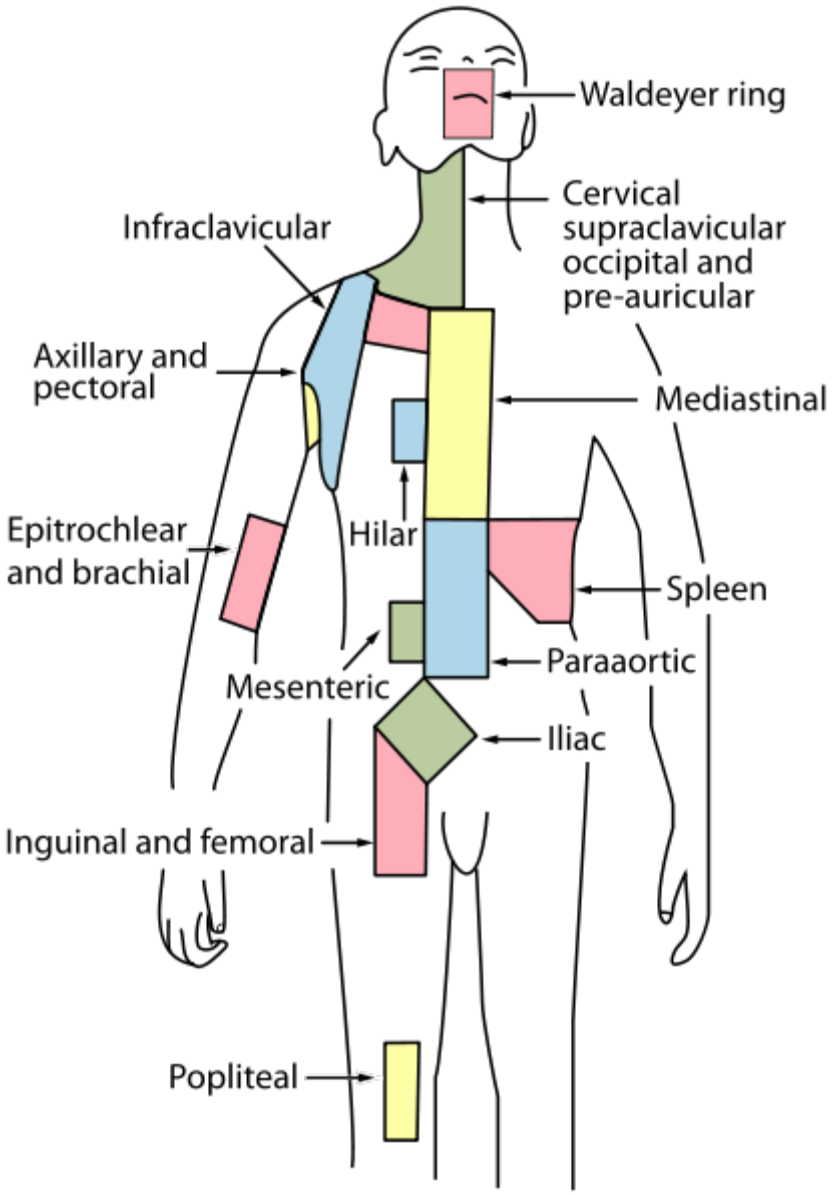
Lymph circulates to the lymph node via *afferent lymphatic vessels* and drains into the node just beneath the capsule in a space called the *subcapsular sinus*. The subcapsular sinus drains into trabecular sinuses and finally into medullary sinuses. The sinus space is criss-crossed by the pseudopods of macrophages, which act to trap foreign particles and filter the lymph. The medullary sinuses converge at the hilum and lymph then leaves the lymph node via the *efferent lymphatic vessel* towards either a more central lymph node or ultimately for drainage into a central venous subclavian blood vessel, most via the postcapillary venules, and cross its wall by the process of diapedesis.

- The B cells migrate to the nodular cortex and medulla.
- The T cells migrate to the deep cortex ("paracortex").

When a lymphocyte recognizes an antigen, B cells become activated and migrate to germinal centers (by definition, a "secondary nodule" has a germinal center, while a "primary nodule" does not). When antibody-producing plasma cells are formed, they migrate to the medullary cords. Stimulation of the lymphocytes by antigens can accelerate the migration process to about 10 times normal, resulting in characteristic swelling of the lymph nodes.

The spleen and tonsils are large lymphoid organs that serve similar functions to lymph nodes, though the spleen filters blood cells rather than lymph.

Distribution



Regional lymph tissue

Humans have approximately 500-600 lymph nodes distributed throughout the body, with clusters found in the underarms, groin, neck, chest, and abdomen.

Lymph nodes of the head and neck

- Cervical lymph nodes
  - Anterior cervical: These nodes, both superficial and deep, lie above and beneath the sternocleidomastoid muscles. They drain the internal

structures of the throat as well as part of the posterior pharynx, tonsils, and thyroid gland.
    - Posterior cervical: These nodes extend in a line posterior to the sternocleidomastoids but in front of the trapezius, from the level of the Mastoid portion of the temporal bone to the clavicle. They are frequently enlarged during upper respiratory tract infections.
- Tonsillar OR Sub-mandibular: These nodes are located just below the angle of the mandible, along the underside of the jaw on either side. They drain the tonsillar and posterior pharyngeal region, including the structures in the floor of the mouth and the maxillary anterior, bicuspid and 1st and 2nd molars. They also drain all of the mandibular teeth except the central incisors.

Retropharyngeal: Drains lymph from the soft palate and the 3rd molars.

- Sub-mental: These nodes are just below the chin. They drain the central incisors and midline of lower lip and tip of the tongue.
- Supraclavicular lymph nodes: These nodes are in the hollow above the clavicle, just lateral to where it joins the sternum. They drain a part of the thoracic cavity and abdomen. Virchow's node is a left supraclavicular lymph node that receives the lymph drainage from most of the body (especially the abdomen) via the thoracic duct and is thus an early site of metastasis for various malignancies.

Lymph nodes of the thorax

- Lymph nodes of the lungs: The lymph is drained from the lung tissue through **subsegmental**, **segmental**, **lobar** and **interlobar** lymph nodes to the **hilar** lymph nodes, which are located around the hilum (the pedicle, which attaches the lung to the mediastinal structures, containing the pulmonary artery, the pulmonary veins, the main bronchus for each side, some vegetative nerves and the lymphatics) of each lung. The lymph flows subsequently to the mediastinal lymph nodes.
- Mediastinal lymph nodes: They consist of several lymph node groups, especially along the trachea (5 groups), along the esophagus and between the lung and the diaphragm. In the mediastinal lymph nodes arises lymphatic ducts, which draines the lymph to the left subclavian vein (to the venous angle in the confluence of the subclavian and deep jugular veins).

The mediastinal lymph nodes along the esophagus are in tight connection with the abdominal lymph nodes along the esophagus and the stomach. That fact facilitates spreading of tumors cells through these lymphatics in cases of cancers of the stomach and particularly of the esophagus. Through the mediastinum, the main lymphatic drainage from the abdominal organs goes via the thoracic duct (*ductus thoracicus*), which drains majority of the lymph from the abdomen to the above mentioned left venous angle.

Lymph nodes of the arm

These drain the whole of the arm, and are divided into two groups, superficial and deep. The superficial nodes are supplied by lymphatics that are present throughout the arm, but are particularly rich on the palm and flexor aspects of the digits.

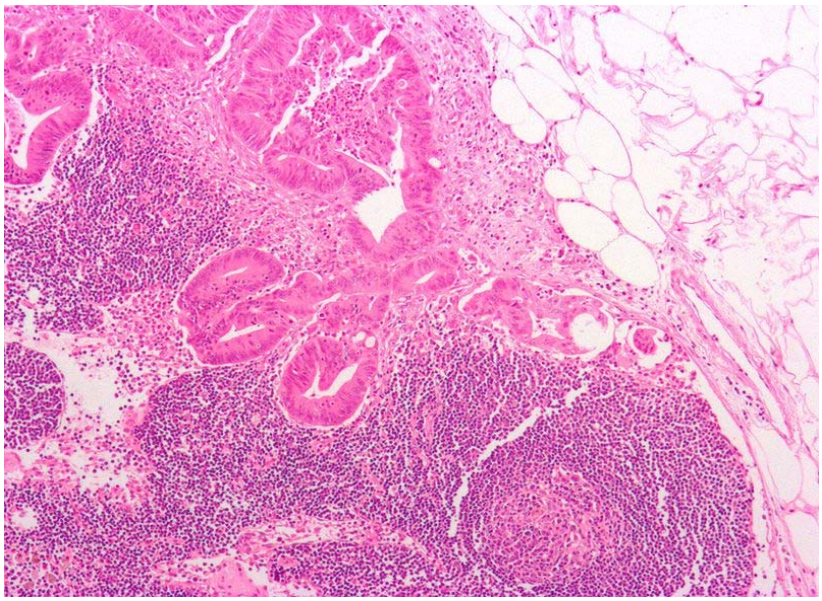
- Superficial lymph glands of the arm:
  - Supratrochlear glands: Situated above the medial epicondyle of the humerus, medial to the basilic vein, they drain the C7 and C8 dermatomes.
  - Deltoideopectoral glands: Situated between the pectoralis major and deltoid muscles inferior to the clavicle.

- Deep lymph glands of the arm: These comprise the axillary glands, which are 20-30 individual glands and can be subdivided into:
  - Lateral glands
  - Anterior or pectoral glands
  - Posterior or subscapular glands
  - Central or intermediate glands
  - Medial or subclavicular glands

Lower limbs

- Superficial inguinal lymph nodes
- Deep inguinal lymph nodes
- Popliteal lymph nodes

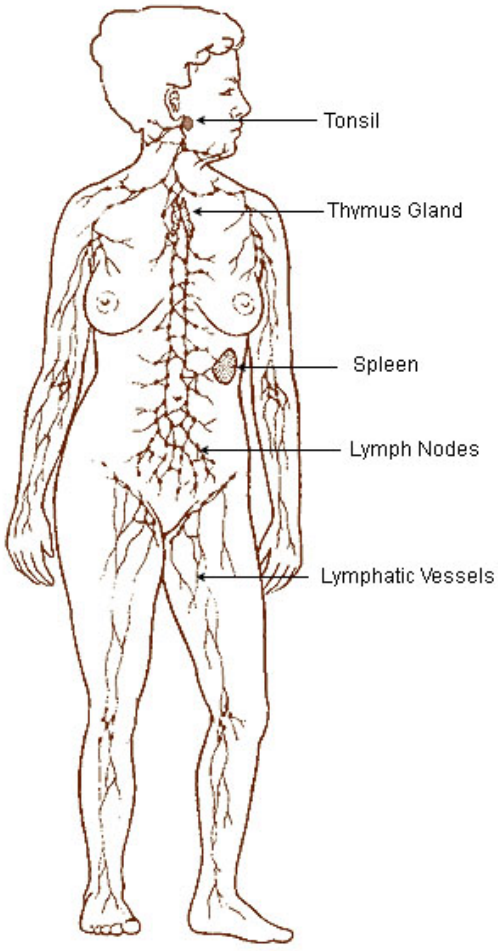
Pathology

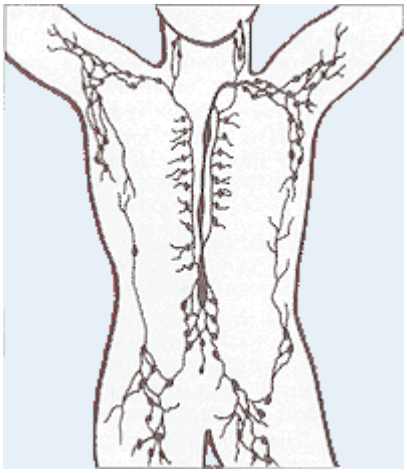


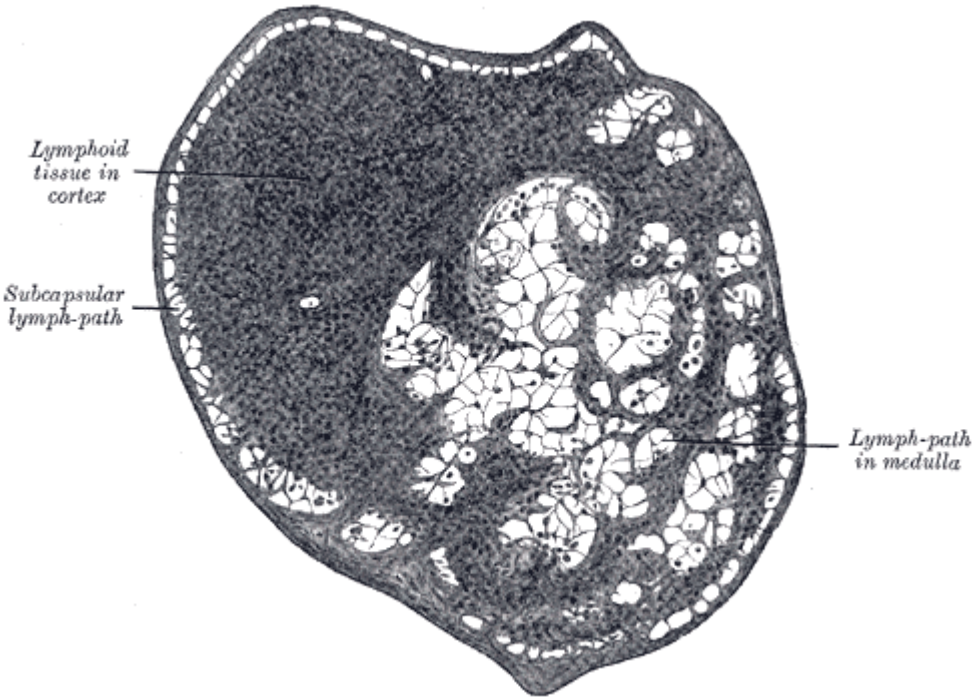
Micrograph of a mesenteric **lymph node** with colorectal adenocarcinoma, the most common type of colorectal cancer.

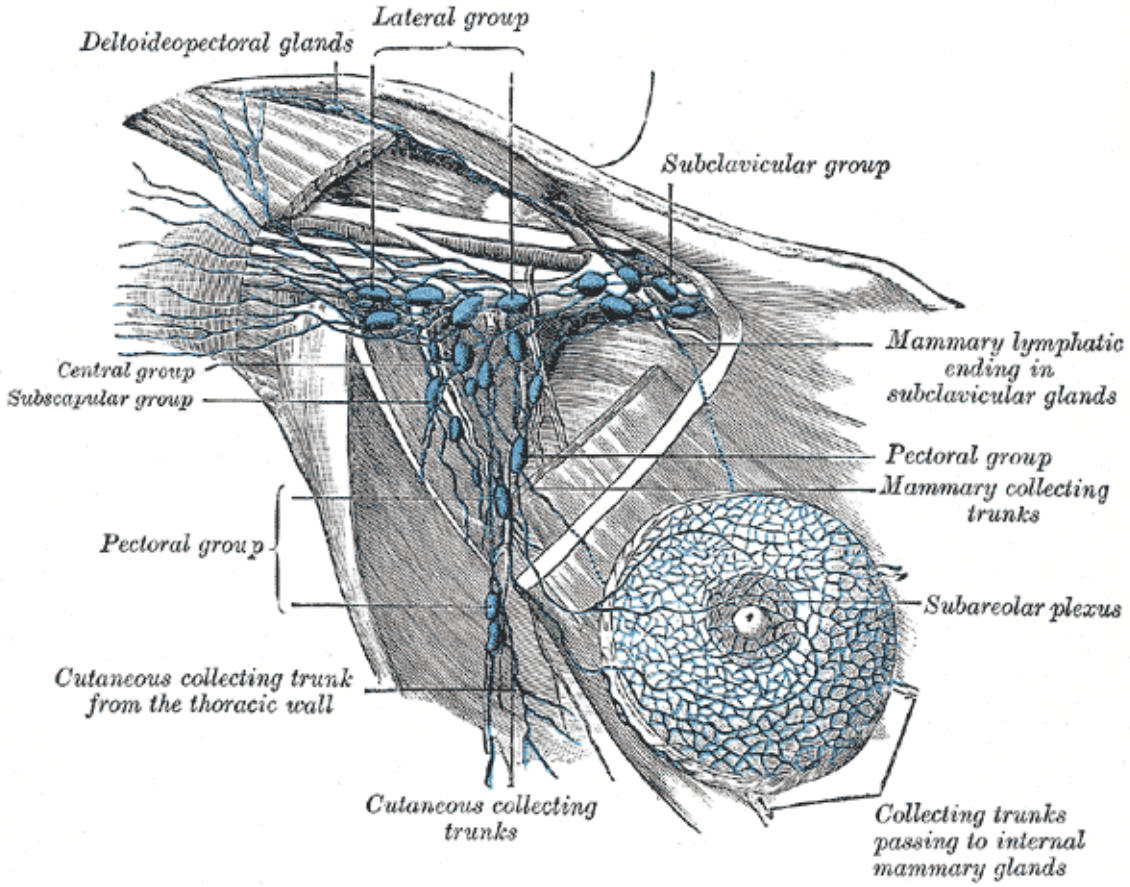
Lymphadenopathy is a term meaning "disease of the lymph nodes." It is, however, almost synonymously used with "swollen/enlarged lymph nodes." In this case, the lymph nodes are palpable, and is a sign of various infections and diseases.

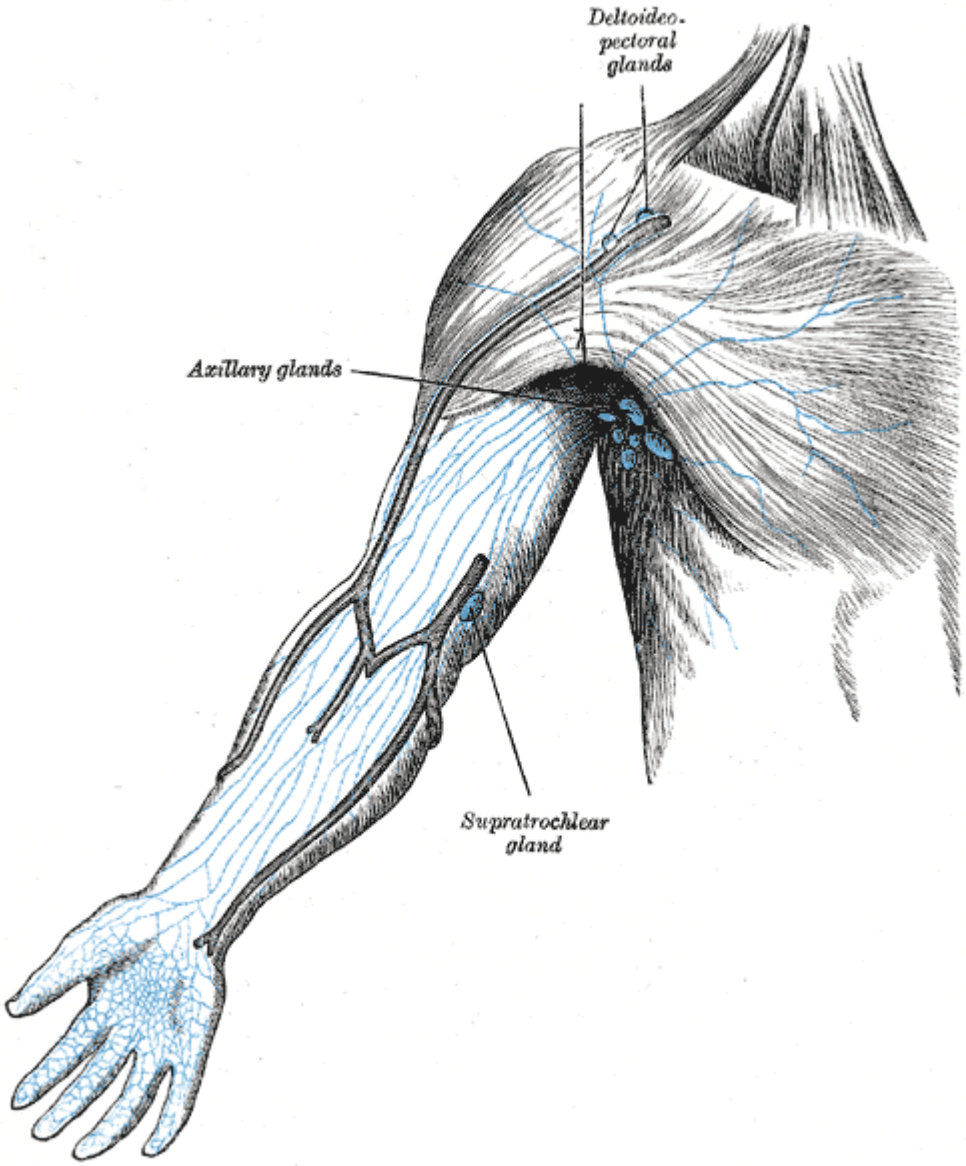
Additional images

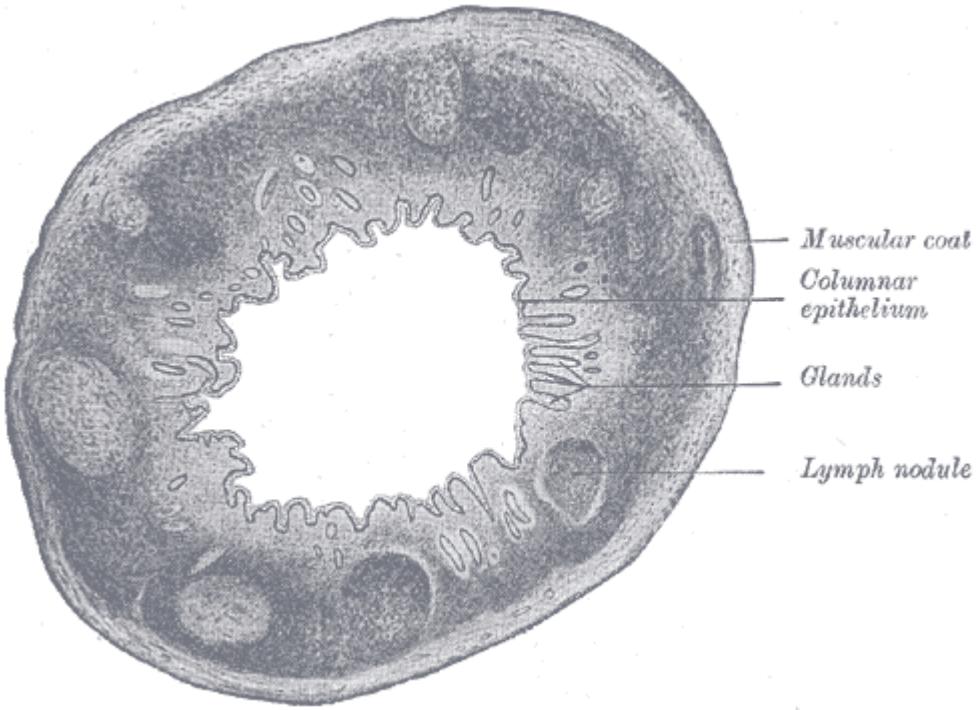


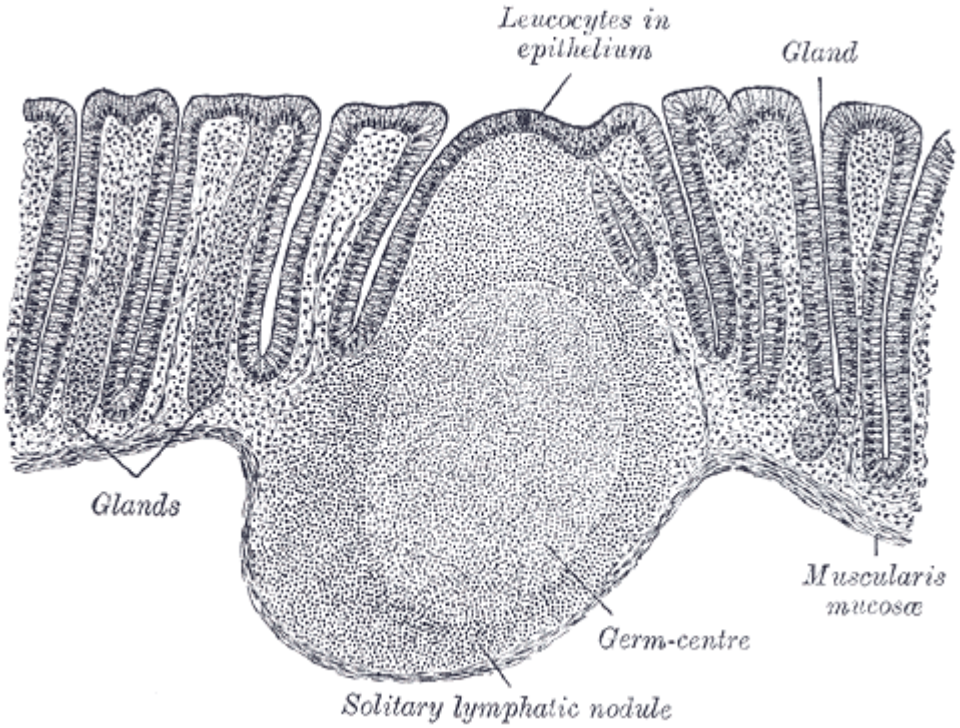



Lymphoid tissue in cortex
Subcapsular lymph-path
Lymph-path in medulla


Deltoideopectoral glands
Lateral group
Subclavicular group
Mammary lymphatic ending in subclavicular glands
Central group
Subscapular group
Pectoral group
Mammary collecting trunks
Pectoral group
Subareolar plexus
Cutaneous collecting trunk from the thoracic wall
Cutaneous collecting trunks
Collecting trunks passing to internal mammary glands


Deltoideo-pectoral glands
Axillary glands
Supratrochlear gland

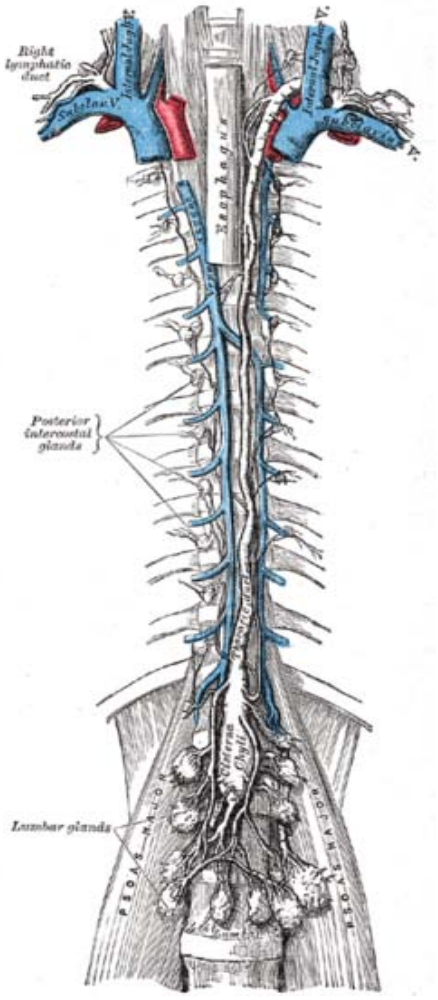

Muscular coat
Columnar epithelium
Glands
Lymph nodule


Leucocytes in epithelium
Gland
Glands
Muscularis mucosæ
Germ-centre
Solitary lymphatic nodule

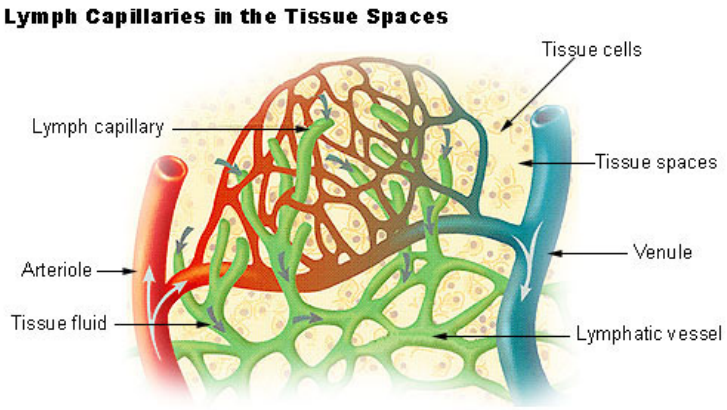
Chapter 3

Lymph Vessel

Lymph vessel



The thoracic duct and right lymphatic duct.



Lymph capillaries in the tissue spaces.

Latin *vas lymphaticum*

In anatomy, **lymph vessels** (or **lymphatic vessels**) are thin walled, valved structures that carry lymph. As part of the lymphatic system, lymph vessels are complementary to the cardiovascular system. Lymph vessels are lined by endothelial cells, and deep to that have a thin layer of smooth muscles, and adventitia that bind the lymph vessel to the surroundings. Lymph vessels are devoted to propulsion of the lymph from the lymph capillaries, which are mainly concerned with absorption of interstitial fluid from the tissues. Lymph capillaries are slightly larger than their counterpart capillaries of the vascular system. Lymph vessel that carries lymph to a lymph node are called the afferent lymph vessel, and one that carries it from a lymph node is called the efferent lymph vessel, from where the lymph may travel to another lymph node or may be returned to a vein, or may travel to a larger lymph duct. Lymph ducts drain the lymph into one of the subclavian veins and thus return it to general circulation.

Generally, lymph flows away from the tissues to lymph nodes and eventually to either the right lymphatic duct or the largest lymph vessel in the body, the thoracic duct. These vessels drain into the right and left subclavian veins respectively.

Function

Lymph vessels act as a reservoir from plasma and other substances including cells that leaked from the vascular system and transport lymph fluid back from the tissues to the circulatory system. Without functioning lymph vessels, lymph cannot be effectively drained and edema typically results.

General structure of Lymphatics

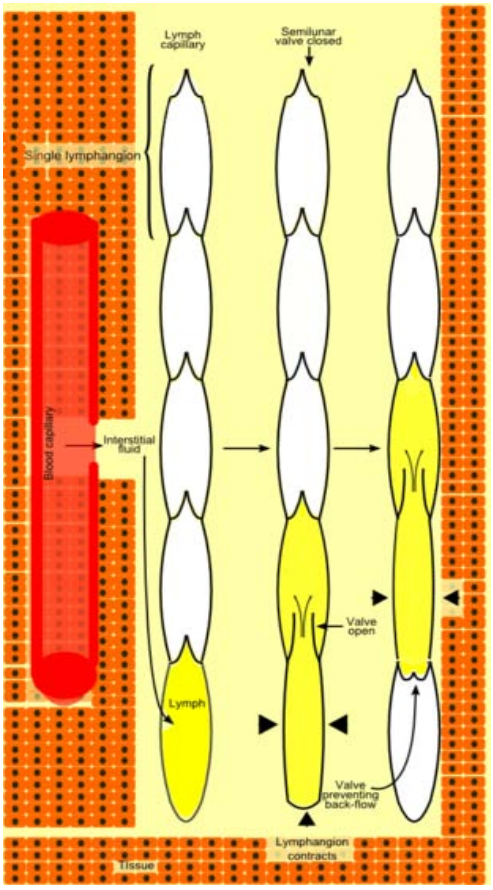
The general structure of lymphatics is based on that of blood vessels. There is an inner lining of single flattened epithelial cells composed of a type of epithelium that is called endothelium, and the cells are called *endothelial cells*. This layer functions to mechanically transport fluid and since the basement membrane on which it rests is

discontinuous; it leaks easily. The next layer is that of smooth muscles that are arranged in a circular fashion around the endothelium, which by shortening (contracting) or relaxing alter the diameter (caliber) of the lumen. The outermost layer is the adventitia that consists of fibrous tissue. The general structure described here is seen only in larger lymphatics; smaller lymphatics have fewer layers. The smallest vessels (*lymphatic* or *lymph capillaries*) lack both the muscular layer and the outer adventitia. As they proceed forward and in their course are joined by other capillaries, they grow larger and first take on an adventitia, and then smooth muscles.

The whole lymphatic conducting system broadly consists of two types of channels—the *initial lymphatics*, the *prelymphatics* or *lymph capillaries* that specialize in collection of the lymph from the ISF, and the larger *lymph vessels* that propel the lymph forward.

Unlike the cardiovascular system, the lymphatic system is not closed and has no central pump. Lymph movement occurs despite low pressure due to peristalsis (propulsion of the lymph due to alternate contraction and relaxation of smooth muscle), valves, and compression during contraction of adjacent skeletal muscle and arterial pulsation.

Lymph capillaries



Propulsion of lymph through lymph vessel

The lymphatic circulation begins with blind ending (closed at one end) highly permeable superficial lymph capillaries, formed by endothelial cells with button-like junctions between them that allow fluid to pass through them when the interstitial pressure is sufficiently high. These button-like junctions consist of protein filaments like platelet endothelial cell adhesion molecule-1 or (PECAM-1). A valve system in place here prevents the absorbed lymph from leaking back into the ISF. There is another system of semilunar (*semi*=half; *lunar*=related to the Moon) valves that prevents back-flow of lymph along the lumen of the vessel. Lymph capillaries have many interconnections (anastomoses) between them and form a very fine network.

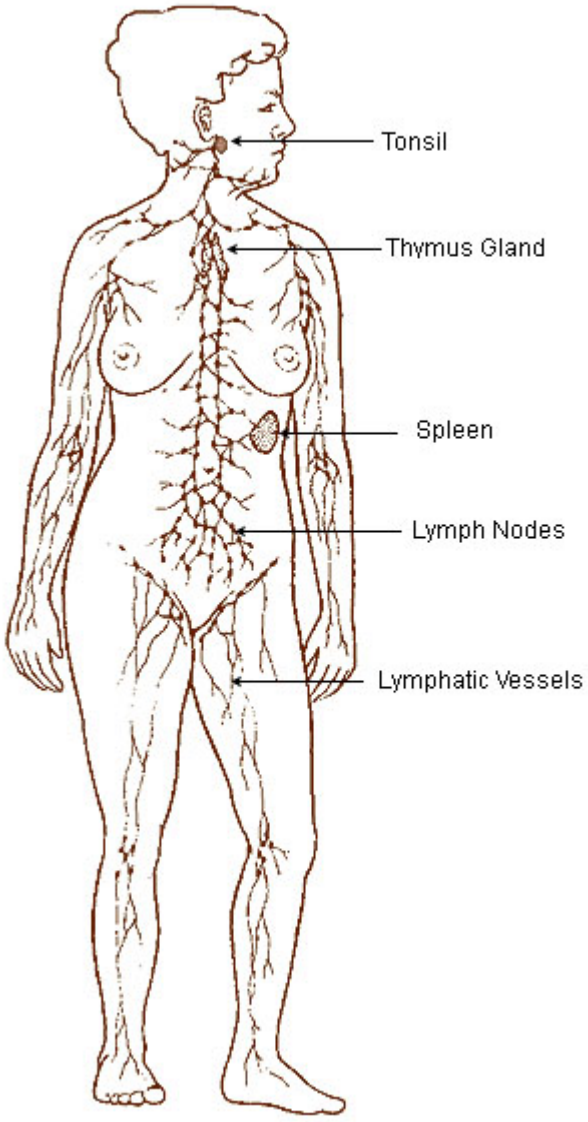
Rhythmic contraction of the vessel walls through movements may also help draw fluid into the smallest lymphatic vessels, capillaries. If tissue fluid builds up the tissue will swell; this is called edema. As the circular path through the body's system continues, the fluid is then transported to progressively larger lymphatic vessels culminating in the right lymphatic duct (for lymph from the right upper body) and the thoracic duct (for the rest of the body); both ducts *drain* into the circulatory system at the right and left subclavian veins. The system collaborates with white blood cells in lymph nodes to protect the body from being infected by cancer cells, fungi, viruses or bacteria. This is known as a secondary circulatory system.

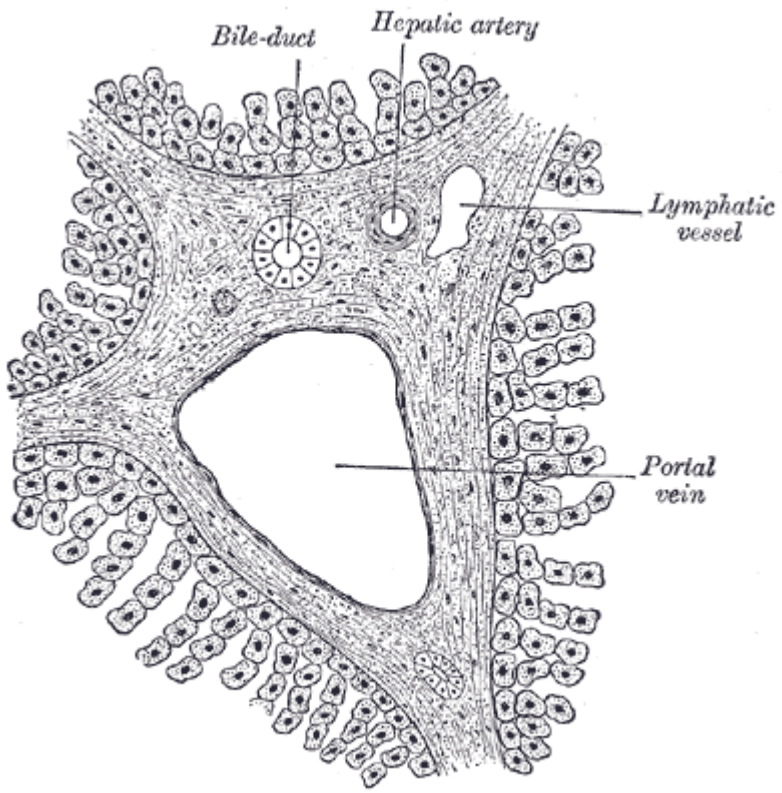
Lymph vessels

The lymph capillaries drain the lymph to larger *contractile* lymphatics, which have valves as well as smooth muscle walls. These are called the *collecting lymphatics*. As the collecting lymph vessel accumulates lymph from more and more lymph capillaries in its course, it becomes larger and is called the afferent lymph vessel as it enters a lymph node. Here the lymph percolates through the lymph node tissue and is removed by the efferent lymph vessel. An efferent lymph vessel may directly drain into one of the (right or thoracic) lymph ducts, or may empty into another lymph node as its afferent lymph vessel. Both the lymph ducts return the lymph to the blood stream by emptying into the subclavian veins

The functional unit of a lymph vessel is known as a *lymphangion*, which is the segment between two valves. Since it is contractile, depending upon the ratio of its length to its radius, it can act either like a contractile chamber propelling the fluid ahead, or as a resistance vessel tending to stop the lymph in its place.

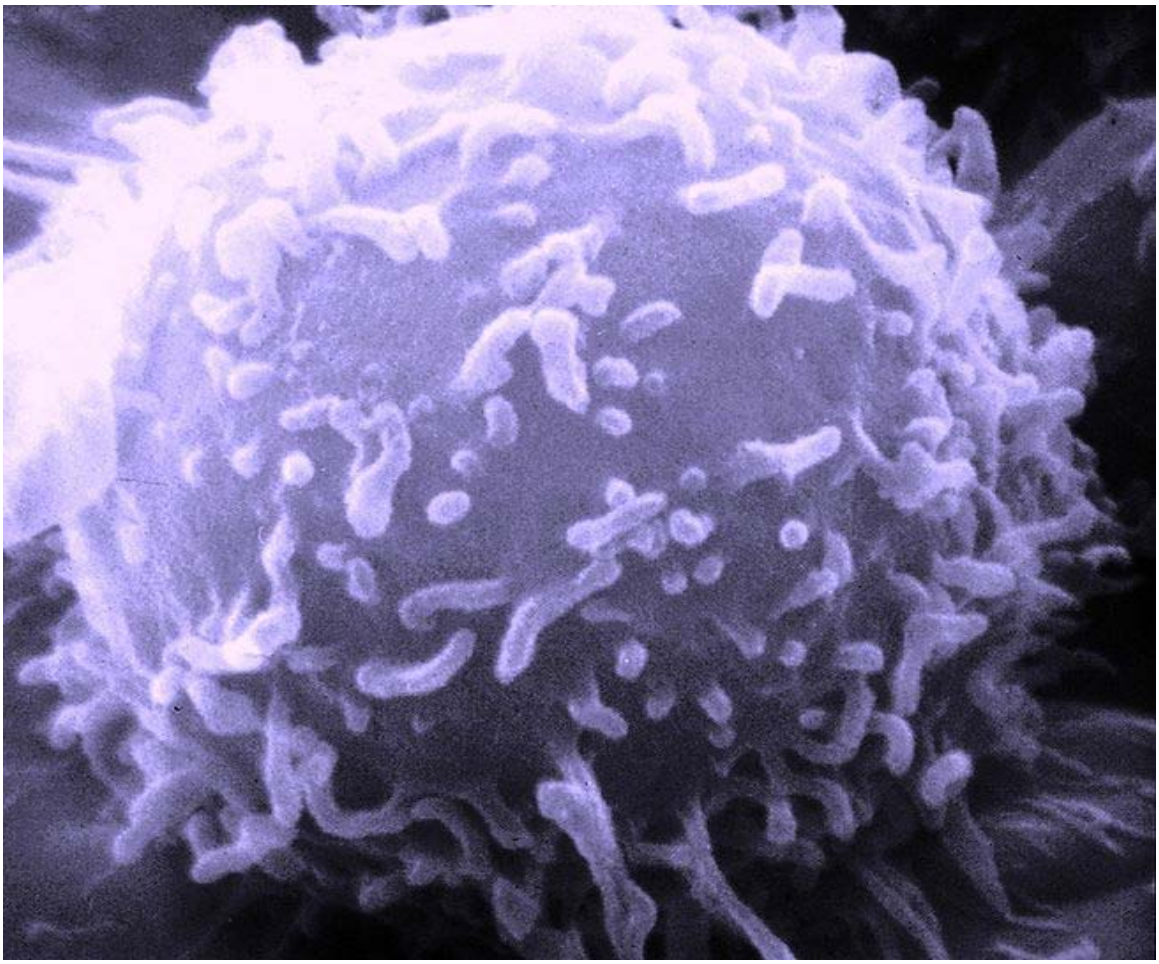
Additional images




Bile-duct
Hepatic artery
Lymphatic vessel
Portal vein

Chapter 4

Lymphocyte

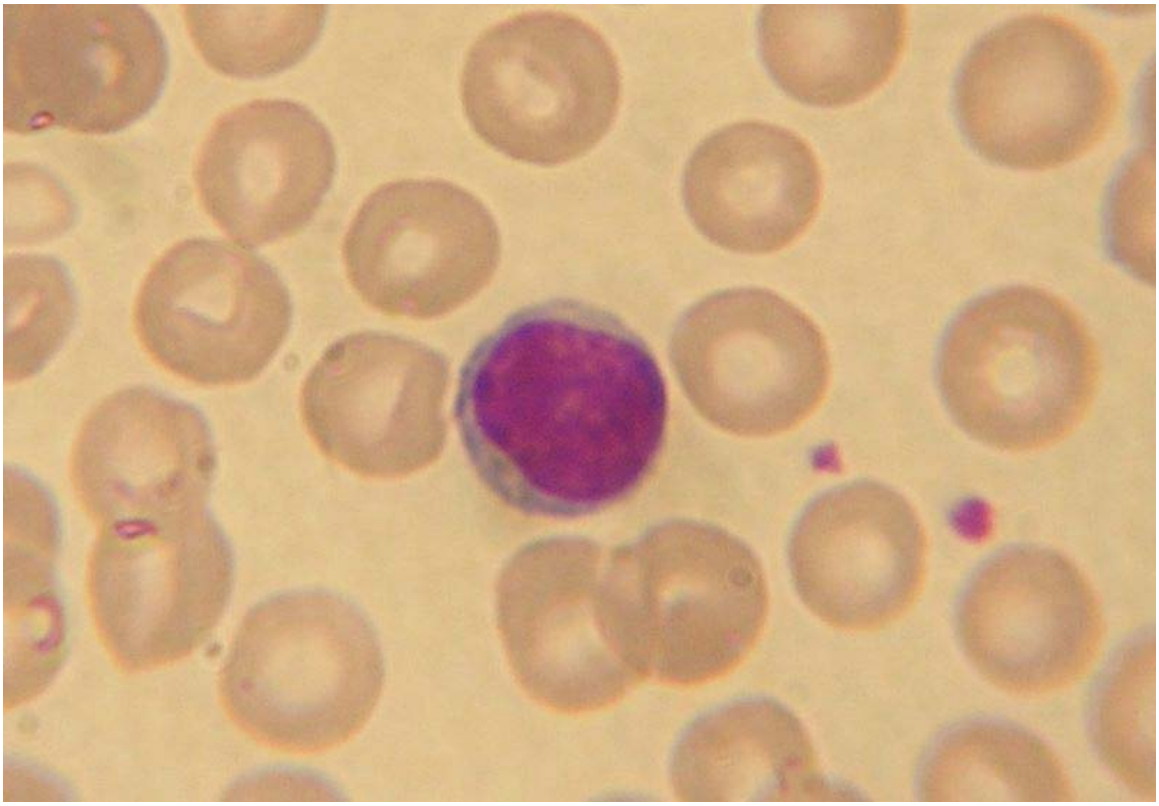


A scanning electron microscope (SEM) image of a single human lymphocyte

A **lymphocyte** is a type of white blood cell in the vertebrate immune system.

Under the microscope, lymphocytes can be divided into large granular lymphocytes and small lymphocytes. Large granular lymphocytes include natural killer cells (NK cells). Small lymphocytes consist of T cells and B cells.

Types



A stained lymphocyte surrounded by red blood cells viewed using a light microscope

The three major types of lymphocyte are T cells, B cells and natural killer (NK) cells.

Natural killer cells

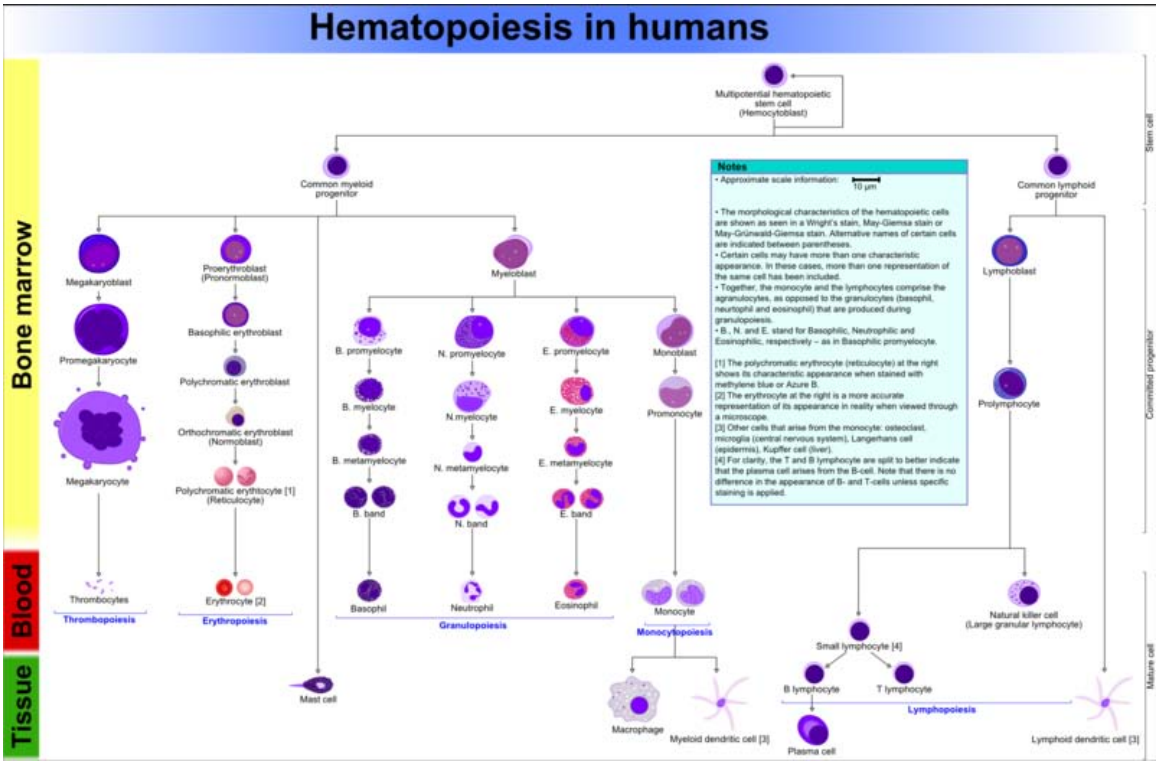
NK cells are a part of innate immune system and play a major role in defending the host from both tumors and virally infected cells. NK cells distinguish infected cells and tumors from normal and uninfected cells by recognizing level changes of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules which then destroy the altered cells. They were named "natural killer cells" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I.

T cells and B cells

T cells (Thymus cells) and B cells (bone cells) are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity whereas B cells are primarily responsible for humoral immunity (relating to antibodies). The function of T cells and B cells is to recognize specific “non-self” antigens, during a process known as antigen presentation. Once they have identified an invader, the cells

generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells. B cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens some T cells, called *T helper cells*, produce cytokines that direct the immune response while other T cells, called *cytotoxic T cells*, produce toxic granules that contain powerful enzymes which induce the death of pathogen infected cells. Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of *memory cells*. Throughout the lifetime of an animal these memory cells will "remember" each specific pathogen encountered, and are able to mount a strong and rapid response if the pathogen is detected again.

Development



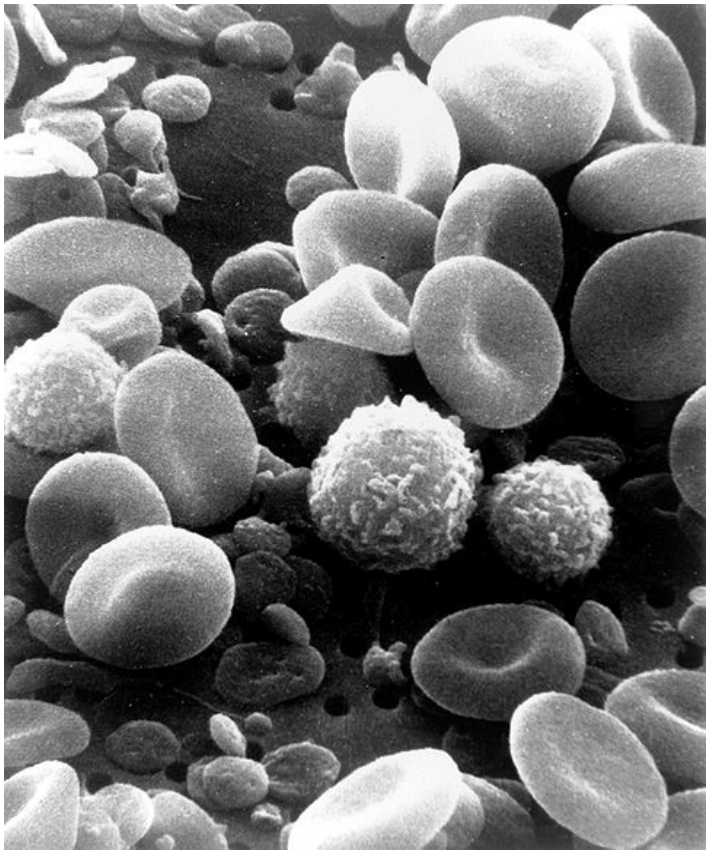
Development of blood cells

Mammalian stem cells differentiate into several kinds of blood cell within the bone marrow. This process is called haematopoiesis. All lymphocytes originate, during this process, from a common lymphoid progenitor before differentiating into their distinct lymphocyte types. The differentiation of lymphocytes follows various pathways in a hierarchical fashion as well as in a more plastic fashion. The formation of lymphocytes is known as lymphopoiesis. B cells mature into B lymphocytes in the bone marrow, while T cells migrate to and mature in a distinct organ, called the thymus. Following maturation, the lymphocytes enter the circulation and peripheral lymphoid organs (e.g. the spleen and lymph nodes) where they survey for invading pathogens and/or tumor cells.

The lymphocytes involved in adaptive immunity (i.e. B and T cells) differentiate further after exposure to an antigen; they form effector and memory lymphocytes. Effector lymphocytes function to eliminate the antigen, either by releasing antibodies (in the case of B cells), cytotoxic granules (cytotoxic T cells) or by signaling to other cells of the immune system (helper T cells). Memory cells remain in the peripheral tissues and circulation for an extended time ready to respond to the same antigen upon future exposure.

They live weeks to several years to a whole lifetime, which is very long compared to other leukocytes.

Characteristics



A scanning electron microscope image of normal circulating human blood showing red blood cells, several types of white blood cells including lymphocytes, a monocyte, a neutrophil and many small disc-shaped platelets.

Microscopically, in a Wright's stained peripheral blood smear, a normal lymphocyte has a large, dark-staining nucleus with little to no eosinophilic cytoplasm. In normal situations, the coarse, dense nucleus of a lymphocyte is approximately the size of a red blood cell (about 7 micrometres in diameter). Some lymphocytes show a clear perinuclear zone (or halo) around the nucleus or could exhibit a small clear zone to one side of the nucleus. Polyribosomes are a prominent feature in the lymphocytes and can be viewed with an

electron microscope. The ribosomes are involved in protein synthesis allowing the generation of large quantities of cytokines and immunoglobulins by these cells.

It is impossible to distinguish between T cells and B cells in a peripheral blood smear. Normally, flow cytometry testing is used for specific lymphocyte population counts. This can be used to specifically determine the percentage of lymphocytes that contain a particular combination of specific cell surface proteins, such as immunoglobulins or cluster of differentiation (CD) markers or that produce particular proteins (for example, cytokines using intracellular cytokine staining (ICCS)). In order to study the function of a lymphocyte by virtue of the proteins it generates, other scientific techniques like the ELISPOT or secretion assay techniques can be used.

Typical recognition markers for lymphocytes

CLASS	FUNCTION	PROPORTION	PHENOTYPIC MARKER(S)
NK cells	Lysis of virally infected cells and tumour cells	7% (2-13%)	CD16 CD56 but not CD3
Helper T cells	Release cytokines and growth factors that regulate other immune cells	46% (28-59%)	TCRαβ, CD3 and CD4
Cytotoxic T cells	Lysis of virally infected cells, tumour cells and allografts	19% (13-32%)	TCRαβ, CD3 and CD8
γδ T cells	Immunoregulation and cytotoxicity		TCRγδ and CD3
B cells	Secretion of antibodies	23% (18-47%)	MHC class II, CD19 and CD21

In the circulatory system they move from lymph node to lymph node. This contrasts with macrophages, which are rather stationary in the nodes.

Lymphocytes and disease

A lymphocyte count is usually part of a peripheral complete blood cell count and is expressed as percentage of lymphocytes to total white blood cells counted.

A general increase in the number of lymphocytes is known as lymphocytosis whereas a decrease is lymphocytopenia.

High

An increase in lymphocyte concentration is usually a sign of a viral infection (in some rare case, leukemias are found through an abnormally raised lymphocyte count in an otherwise normal person).

Low

A low normal to low absolute lymphocyte concentration is associated with increased rates of infection after surgery or trauma.

One basis for low T cell lymphocytes occurs when the human immunodeficiency virus (HIV) infects and destroys T cells (specifically, the $CD4^+$ subgroup of T lymphocytes). Without the key defense that these T cells provide, the body becomes susceptible to opportunistic infections that otherwise would not affect healthy people. The extent of HIV progression is typically determined by measuring the percentage of $CD4^+$ T cells in the patient's blood. The effects of other viruses or lymphocyte disorders can also often be estimated by counting the numbers of lymphocytes present in the blood.

Tumor-Infiltrating Lymphocytes

In some cancers, eg. melanoma and colorectal cancer, lymphocytes can migrate into and attack the tumor. This can sometimes lead to regression of the primary tumor.

Chapter 5

Bone Marrow

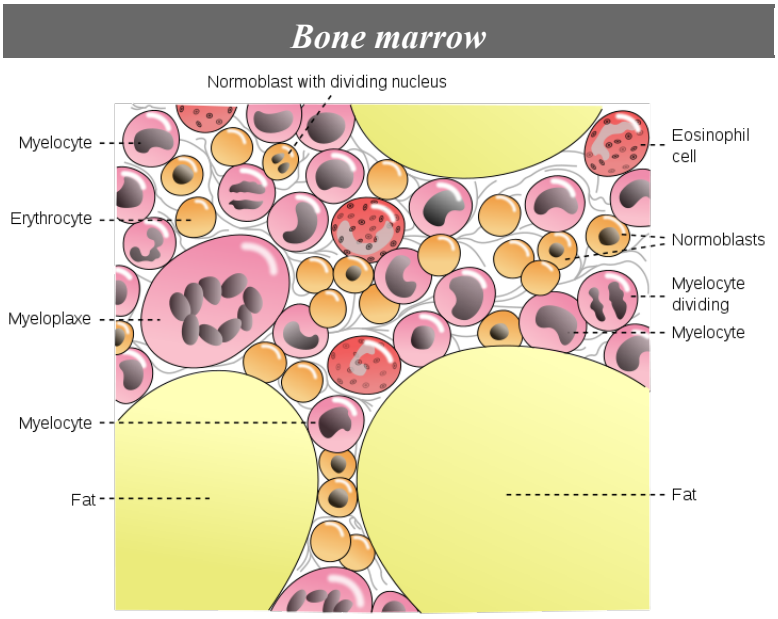


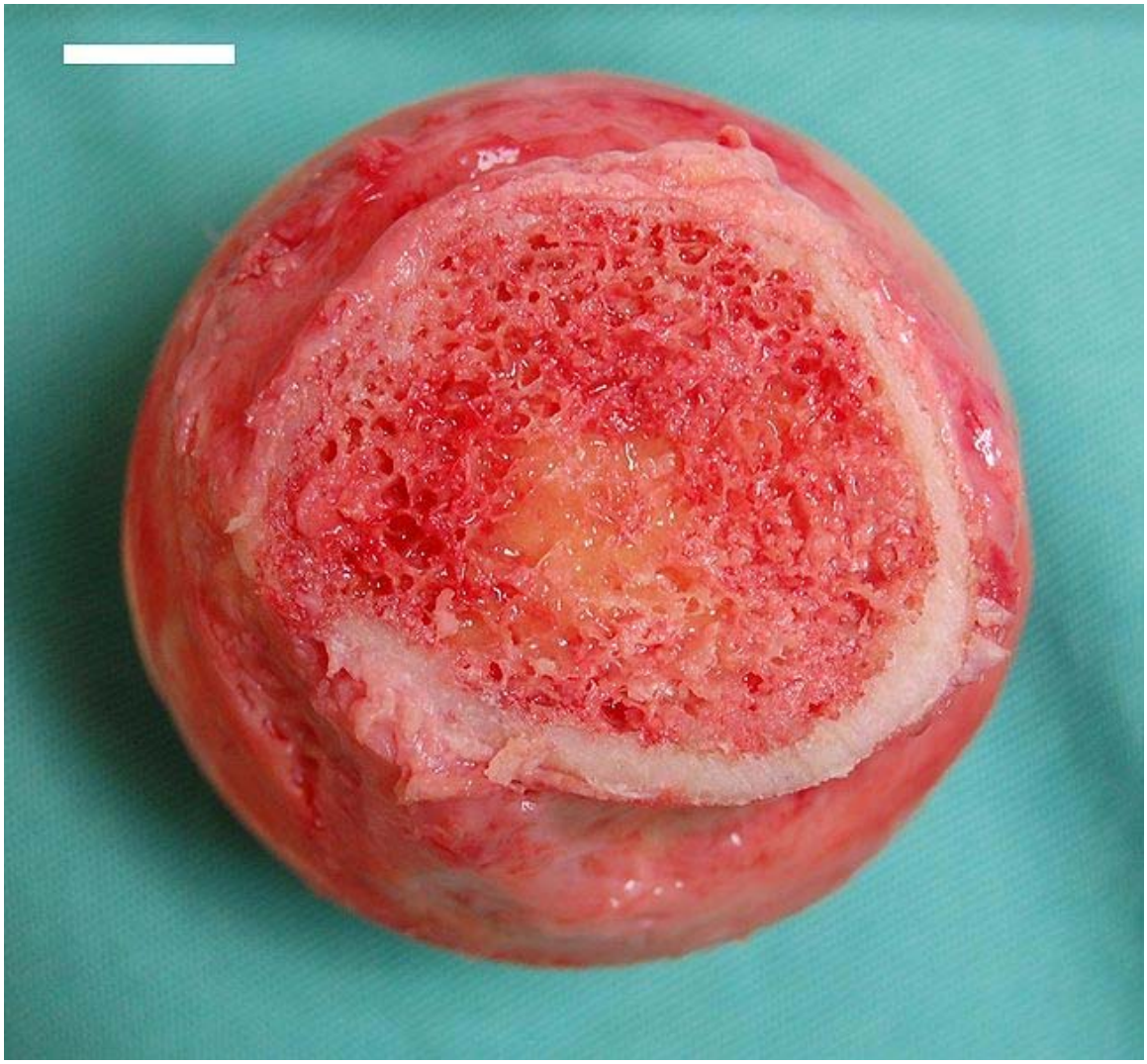
Illustration of cells in bone marrow

Latin *medulla ossium*

Bone marrow is the flexible tissue found in the interior of bones. In humans, marrow in large bones produces new blood cells. It constitutes 4% of the total body weight of humans, i.e. approximately 2.6 kg (5.7 lbs.) in adults. Bone marrow also prevents the backflow of lymph, working as a vital part of the lymphatic system.

Anatomy

Marrow types



A femur with a cortex of cortical bone and medulla of trabecular bone showing its red bone marrow and a focus of yellow bone marrow.

There are two types of bone marrow: **red marrow** (consisting mainly of hematopoietic tissue) and **yellow marrow** (consisting mainly of fat cells). Red blood cells, platelets and most white blood cells arise in red marrow. Both types of bone marrow contain numerous blood vessels and capillaries.

At birth, all bone marrow is red. With age, more and more of it is converted to the yellow type. About half of adult bone marrow is red. Red marrow is found mainly in the flat bones, such as the hip bone, breast bone, skull, ribs, vertebrae and shoulder blades, and in the cancellous ("spongy") material at the epiphyseal ends of the long bones such as the

femur and humerus. Yellow marrow is found in the hollow interior of the middle portion of long bones.

In cases of severe blood loss, the body can convert yellow marrow back to red marrow to increase blood cell production.

Stroma

The *stroma of the bone marrow* is all tissue not directly involved in the primary function of hematopoiesis. The yellow bone marrow belongs here, and makes the majority of the bone marrow stroma, in addition to stromal cells located in the red bone marrow. Yellow bone marrow is found in the Medullary cavity.

Still, the stroma is indirectly involved in hematopoiesis, since it provides the *hematopoietic microenvironment* that facilitates hematopoiesis by the parenchymal cells. For instance, they generate colony stimulating factors, affecting hematopoiesis.

Cells that constitute the bone marrow stroma are:

- fibroblasts (reticular connective tissue)
- macrophages
- adipocytes
- osteoblasts
- osteoclasts
- endothelial cells forming the sinusoids

Macrophages contribute especially to red blood cell production. They deliver iron for hemoglobin-production.

Bone marrow barrier

The blood vessels constitute a barrier, inhibiting immature blood cells from leaving the bone marrow. Only mature blood cells contain the membrane proteins required to attach to and pass the blood vessel endothelium.

Hematopoietic stem cells may also cross the bone marrow barrier, and may thus be harvested from blood.

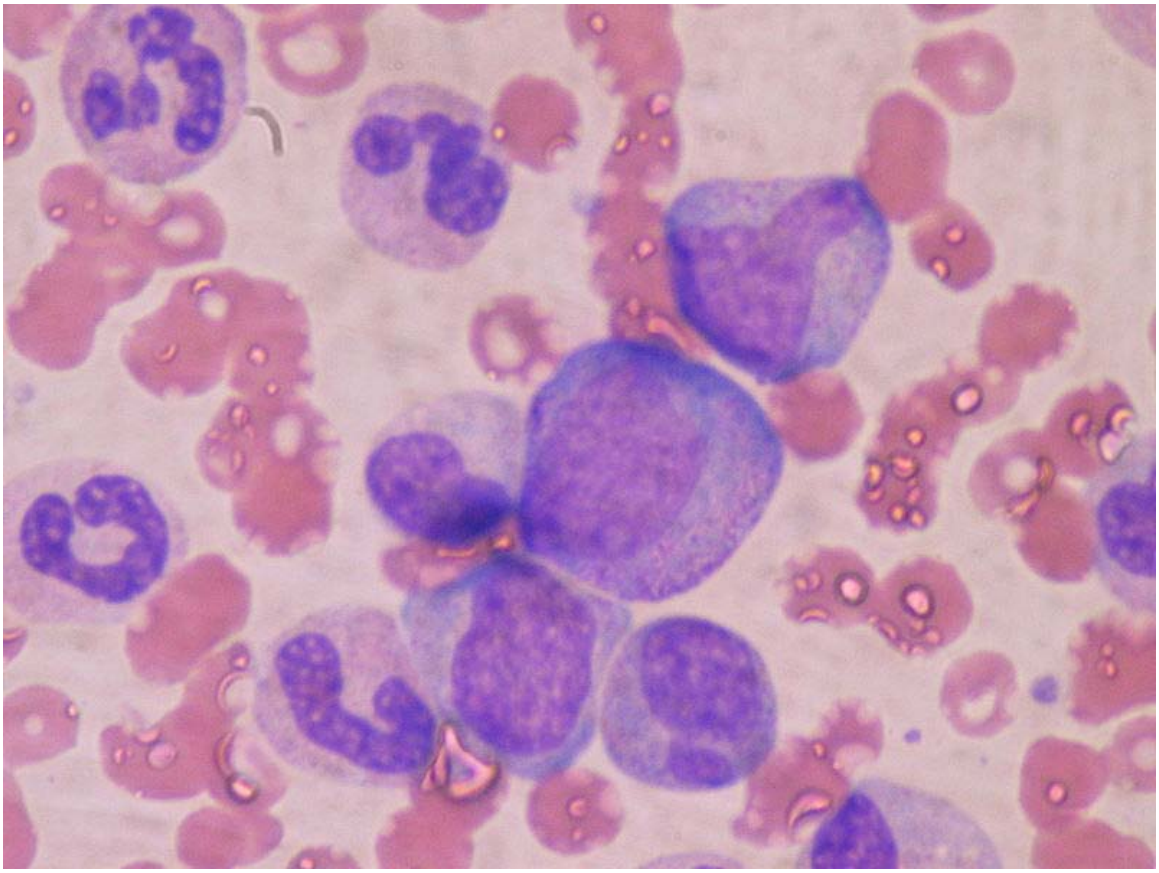
Stem cells

The bone marrow stroma contain *mesenchymal stem cells* (also called *marrow stromal cells*). These cells are multipotent stem cells that can differentiate into a variety of cell types. Cell types that MSCs have been shown to differentiate into in vitro or in vivo include osteoblasts, chondrocytes, myocytes, adipocytes, and, as described lately, beta-pancreatic islets cells. They can also transdifferentiate into neuronal cells.

Compartmentalization

There is biologic compartmentalization in the bone marrow, in that certain cell types tend to aggregate in specific areas. For instance, erythrocytes, macrophages and their precursors tend to gather around blood vessels, while granulocytes gather at the borders of the bone marrow.

Types of stem cells



Hematopoietic precursor cells: promyelocyte in the center, two metamyelocytes next to it and band cells from a bone marrow aspirate.

Bone marrow contains three types of stem cells:

- Hematopoietic stem cells give rise to the three classes of blood cells that are found in the circulation: white blood cells (leukocytes), red blood cells (erythrocytes), and platelets (thrombocytes).
- Mesenchymal stem cells are found arrayed around the central sinus in the bone marrow. They have the capability to differentiate into osteoblasts, chondrocytes, myocytes, and many other types of cells. They also function as "gatekeeper" cells of the bone marrow.
- Endothelial stem cells

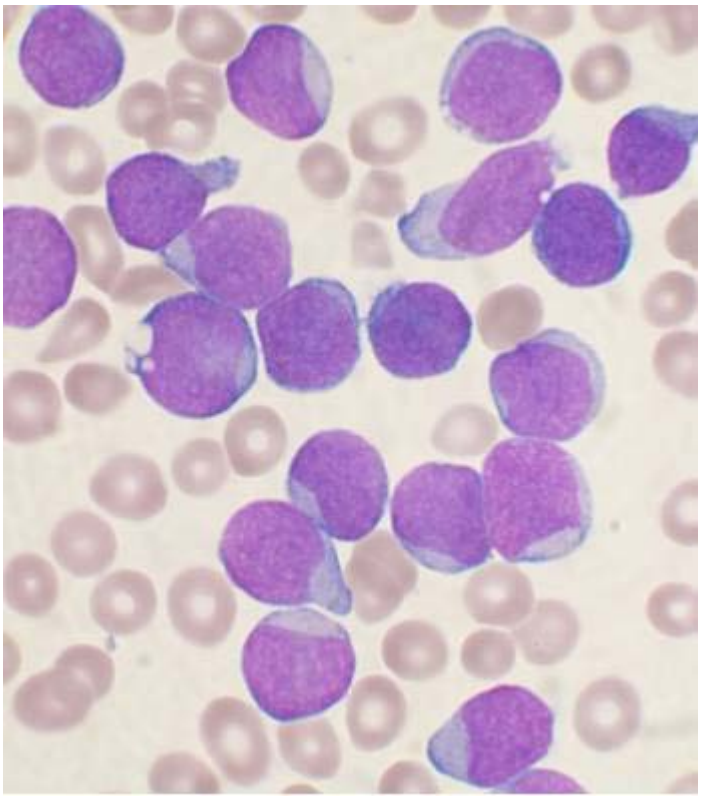
Diseases involving the bone marrow

The normal bone marrow architecture can be displaced by malignancies, aplastic anemia, or infections such as tuberculosis, leading to a decrease in the production of blood cells and blood platelets. In addition, cancers of the hematologic progenitor cells in the bone marrow can arise; these are the leukemias.

To diagnose diseases involving the bone marrow, a bone marrow aspiration is sometimes performed. This typically involves using a hollow needle to acquire a sample of red bone marrow from the crest of the ilium under general or local anesthesia. The average number of cells in a leg bone is about 440,000,000,000 ($440x10^9$).

Exposure to radiation or chemotherapy will kill many of the rapidly dividing cells of the bone marrow and will therefore result in a depressed immune system. Many of the symptoms of radiation sickness are due to damage to the bone marrow cells.

Examination

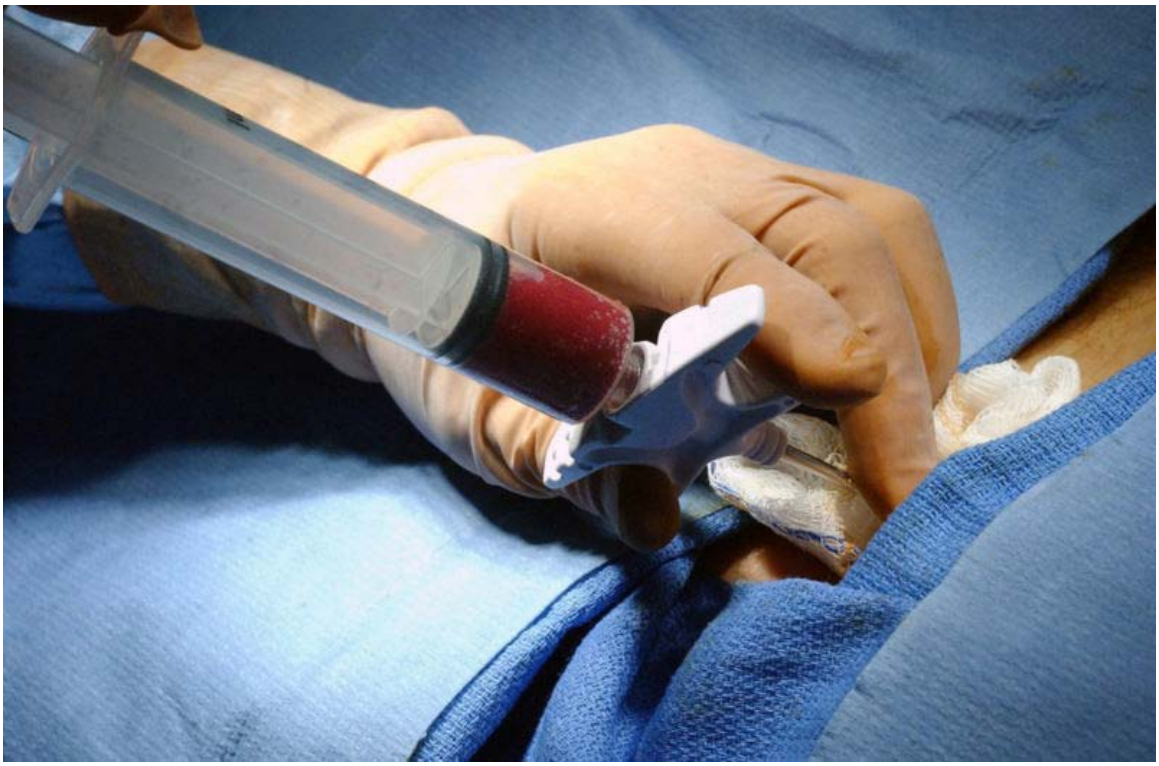


A Wright's stained bone marrow aspirate smear from a patient with leukemia

Bone marrow examination is the pathologic analysis of samples of bone marrow obtained by *bone marrow biopsy* and *bone marrow aspiration*. Bone marrow examination is used in the diagnosis of a number of conditions, including leukemia, multiple myeloma, anemia, and pancytopenia. The bone marrow produces the cellular elements of the blood,

including platelets, red blood cells and white blood cells. While much information can be gleaned by testing the blood itself (drawn from a vein by phlebotomy), it is sometimes necessary to examine the source of the blood cells in the bone marrow to obtain more information on hematopoiesis; this is the role of bone marrow aspiration and biopsy.

Donation and transplantation of bone marrow



Bone marrow harvest

It is possible to take hematopoietic stem cells from one person and then infuse them into another person (Allogenic) or into the same person at a later time (Autologous). If donor and recipient are compatible, these infused cells will then travel to the bone marrow and initiate blood cell production.

Transplantation from one person to another is performed in severe cases of disease of the bone marrow. The patient's marrow is first killed off with drugs or radiation, and then the new stem cells are introduced.

Before radiation therapy or chemotherapy in cases of cancer, some of the patient's hematopoietic stem cells are sometimes harvested and later infused back when the therapy is finished to restore the immune system.

Harvesting

The stem cells are harvested directly from the red marrow in the crest of the ilium, often under general anesthesia. The procedure is minimally invasive and does not require stitches afterwards. Depending on the donor health and reaction to the procedure, the actual harvesting can be an outpatient procedure or requiring 1–2 days of recovery in the hospital. Another option is to administer certain drugs that stimulate the release of stem cells from the bone marrow into circulating blood. An IV is inserted into the donor's arm, and the stem cells are filtered out of the blood. The procedure is similar to donating blood or platelets.

It may also be taken from the sternum. The tibia may seem a good source, since it is very superficial. However, except in children, this bone marrow does not contain any substantial amount of red bone marrow, only yellow bone marrow.

In newborns, stem cells may be retrieved from the umbilical cord.

Bone marrow as a food

In some parts of Germany beef soup is served with "Markklößchen" (bone marrow balls)

Many cultures utilize bone marrow as a food. The Vietnamese prize beef bone as the soup base for their national staple *phở*; Alaskan Natives eat the bone marrow of caribou and moose; Indians use slow-cooked marrow as the core ingredient of the Indian dish nalli nihari; Mexicans use beef bone marrow from leg bones, called tuétano, which is cooked and served as filling for tacos or tostadas; it is also considered to be the highlight of the Italian dish ossobuco (braised veal shanks); beef marrowbones are often included in the French pot-au-feu broth, the cooked marrow being traditionally eaten on toasted bread with sprinkled coarse sea salt, in Iranian cuisine lamb shanks are usually broken before cooking to allow diners to suck out and eat the marrow when the dish is served. Though once used in various preparations, including pemmican, bone marrow has fallen out of favor as a food in the United States, though bone marrow is used in many gourmet restaurants and is popular among foodies. In the Philippines, the soup "Bulalo" is made primarily of beef stock and marrow bones, seasoned with vegetables and boiled meat. In Hungary tibia is a main ingredient of beef soup; the bone is chopped into short (10–15 cm) pieces and the ends are covered with salt to prevent the marrow from leaving the bone while cooking. Upon serving the soup the marrow is usually spread on toast.

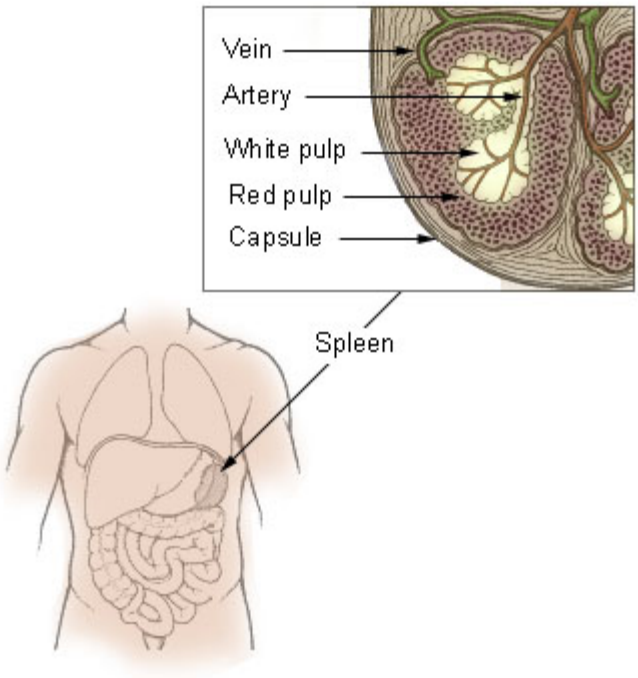
Diners in the 18th century used a marrow scoop (or marrow spoon), often of silver and with a long thin bowl, as a table implement for removing marrow from a bone.

Some anthropologists believe that early humans were scavengers rather than hunters. Marrow would then have been a major protein source for tool-using hominids, who were able to crack open the bones of carcasses left by top predators such as lions.

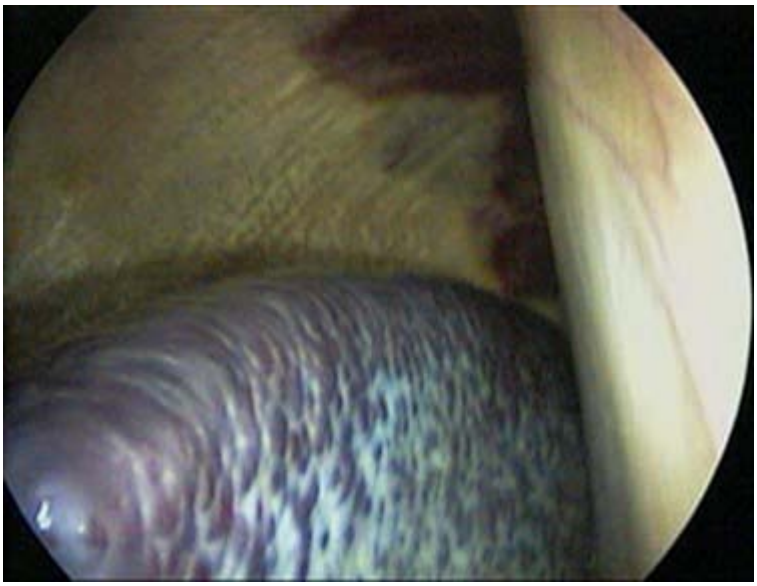
Chapter 6

Spleen

Spleen



Spleen



Laparoscopic view of a horse's spleen (the purple and grey mottled organ)

Latin	*splen, lien*

Artery	Splenic artery
Vein	Splenic vein
Nerve	Splenic plexus
Precursor	Mesenchyme of dorsal mesogastrium
MeSH	*Spleen*
Dorlands/Elsevier	*Spleen*

The **spleen** (from Greek σπλήν - *splēn*) is an organ found in virtually all vertebrate animals with important roles in regard to red blood cells and the immune system. In humans, it is located in the left upper quadrant of the abdomen. It removes old red blood cells and holds a reserve of blood in case of hemorrhagic shock while also recycling iron. It synthesizes antibodies in its white pulp and removes antibody-coated bacteria along with antibody-coated blood cells by way of blood and lymph node circulation. The spleen is purple and gray. Recently, it has been found to contain in its reserve half of the body's monocytes within the red pulp. These monocytes, upon moving to injured tissue (such as the heart), turn into dendritic cells and macrophages while promoting tissue healing. It is one of the centers of activity of the reticuloendothelial system and can be considered analogous to a large lymph node, as its absence leads to a predisposition toward certain infections.

Anatomy

The spleen, in healthy adult humans, is approximately 11 centimetres (4.3 in) in length. It usually weighs between 150 grams (5.3 oz) and 200 grams (7.1 oz) and lies beneath the 9th to the 12th thoracic ribs.

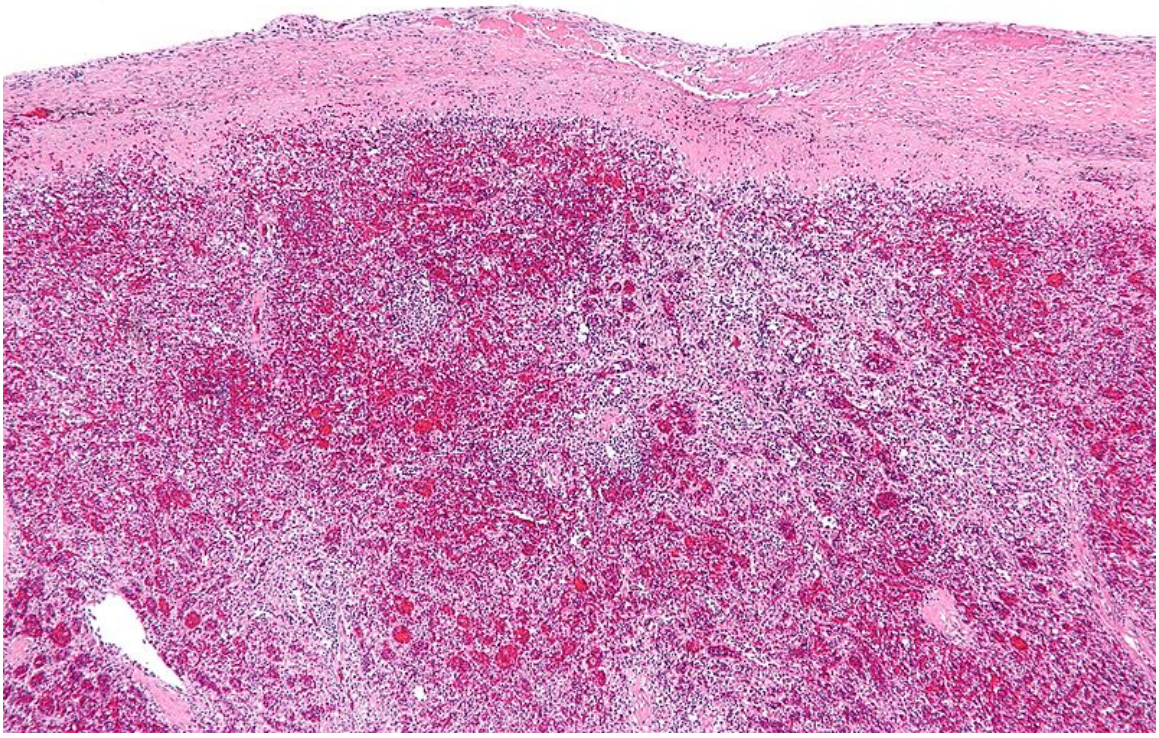
Like the thymus, the spleen possesses only efferent lymphatic vessels.

The spleen is part of the lymphatic system.

The germinal centers are supplied by arterioles called *penicilliary radicles*.

The spleen is unique in respect to its development within the gut. While most of the gut viscera are endodermally derived (with the exception of the neural-crest derived suprarenal gland), the spleen is derived from mesenchymal tissue. Specifically, the spleen forms within, and from, the dorsal mesentery. However, it still shares the same blood supply — the celiac trunk — as the foregut organs.

Function



Micrograph of splenic tissue showing the red pulp (red), white pulp (blue) and a thickened inflamed capusule (mostly pink - top of image). H&E stain.

Area	Function	Composition
red pulp	Mechanical filtration of red blood cells. In mice: Reserve of monocytes	• "sinuses" (or "sinusoids") which are filled with blood • "splenic cords" of reticular fibers • "marginal zone" bordering on white pulp
white pulp	Active immune response through humoral and cell-mediated pathways.	Composed of nodules, called Malpighian corpuscles. These are composed of: • "lymphoid follicles" (or "follicles"), rich in B-lymphocytes • "periarteriolar lymphoid sheaths" (PALS), rich in T-lymphocytes

Other functions of the spleen are less prominent, especially in the healthy adult:

- Production of opsonins, properdin, and tuftsin.
- Creation of red blood cells. While the bone marrow is the primary site of hematopoiesis in the adult, the spleen has important hematopoietic functions up until the fifth month of gestation. After birth, erythropoietic functions cease, except in some hematologic disorders. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes and, as such, remains an hematopoietic organ.
- Storage of red blood cells and other formed elements. In horses roughly 30% of the red blood cells are stored there. The red blood cells can be released when needed. In humans, it does not act as a reservoir of blood cells. It can also store platelets in case of an emergency.
- In mice, the spleen stores half the body's monocytes so that upon injury they can migrate to the injured tissue and transform into dendritic cells and macrophages and so assist wound healing.

Effect of removal

Surgical removal causes:

- modest increases in circulating white blood cells and platelets,
- diminished responsiveness to some vaccines,
- increased susceptibility to infection by bacteria and protozoa; in particular, there is an increased risk of sepsis from polysaccharide encapsulated bacteria.

A 28-year follow-up of 740 veterans of World War II who had their spleen removed on the battlefield found that those who had been splenectomised showed a significant excess of mortality from pneumonia (6 rather than the expected 1.3) and a significant excess of

mortality from ischaemic heart disease (4.1 rather than the expected 3) but not from other conditions.

Disorders

Disorders include splenomegaly, where the spleen is enlarged for various reasons, and asplenia, where the spleen is not present or functions abnormally.

Etymology and cultural views

The word **spleen** comes from the Greek σπλήν, and is the idiomatic equivalent of the heart in English, i.e. to be good-spleened (εὔσπλαγχνος) means to be good-hearted or compassionate.

In English the word *spleen* was customary during the period of the 18th century. Authors like Richard Blackmore or George Cheyne employed it to characterize the hypocondriacal and hysterical affections.

In French, "splénétique" refers to a state of pensive sadness or melancholy. It has been popularized by the poet Charles Baudelaire (1821–1867) but was already used before in particular to the Romantic literature (18th century). The word for the organ is "la rate."

The connection between *spleen* (the organ) and *melancholy* (the temperament) comes from the humoral medicine of the ancient Greeks. One of the humours (body fluid) was the black bile, secreted by the spleen organ and associated with melancholy. In contrast, the Talmud (tractate Berachoth 61b) refers to the spleen as the organ of laughter while possibly suggesting a link with the humoral view of the organ. In the eighteenth- and nineteenth-century England, women in bad humour were said to be afflicted by the spleen, or the vapours of the spleen. In modern English, "to vent one's spleen" means to vent one's anger, e.g. by shouting, and can be applied to both males and females. Similarly, the English term "splenetic" is used to describe a person in a foul mood.

In Chinese, the spleen '脾 (pí)' counts as the seat of one's temperament and is thought to influence the individual's willpower. Analogous to "venting one's spleen", "發脾氣" is used as an expression for getting angry, although in the view of Traditional Chinese Medicine, the view of "脾" does not correspond to the anatomical "spleen". "脾" is a conceptual functional group that mainly has regards to digestion which, in some scholars' opinions, corresponds to the function of the pancreas.

Variation among vertebrates

In cartilagenous and ray-finned fish the spleen is normally a somewhat elongated organ, consisting primarily of red pulp, with only a small amount of white pulp. In lungfish, the spleen is not a distinct organ as it actually lies inside the serosal lining of the intestine. In

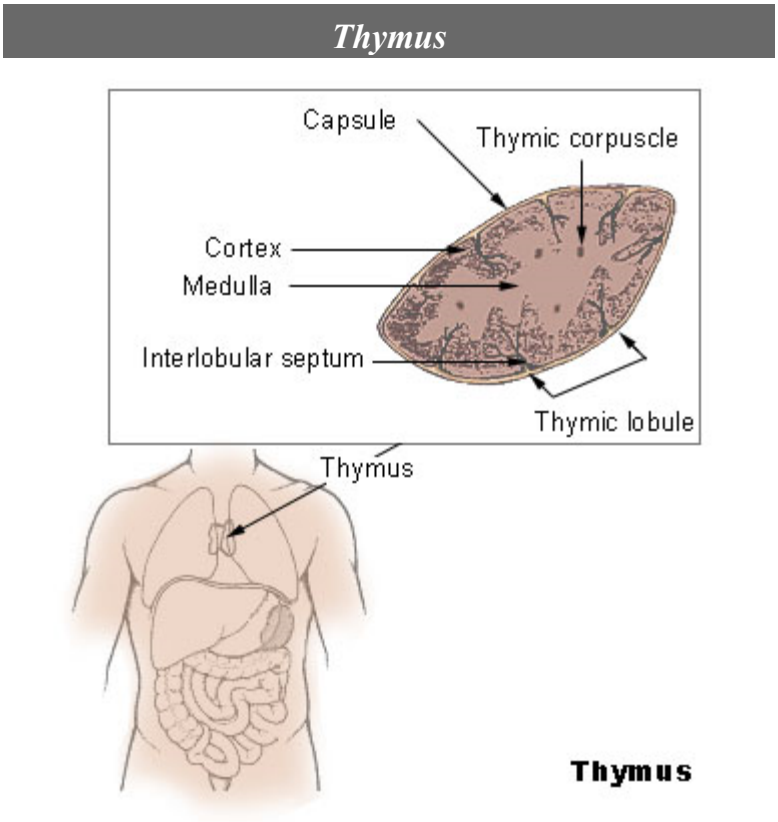
many amphibians, especially frogs, it takes on the more rounded form and there is often a greater quantity of white pulp.

In reptiles, birds, and mammals, white pulp is always relatively plentiful, and in the latter two groups, the spleen is typically rounded, although it adjusts its shape somewhat to the arrangement of the surrounding organs. In the great majority of vertebrates, the spleen continues to produce red blood cells throughout life; it is only in mammals that this function is lost in the adult. Many mammals possess tiny spleen-like structures known as **haemal nodes** throughout the body, which presumably have the same function as the spleen proper. The spleens of aquatic mammals are in some ways dissimilar to those of fully land dwelling mammals. In general the spleens of aquatic mammals are bluish in colour. In cetaceans and manatees it tends to be quite small, but in deep diving pinnipeds it can be quite massive, owing to its function of storing red blood cells.

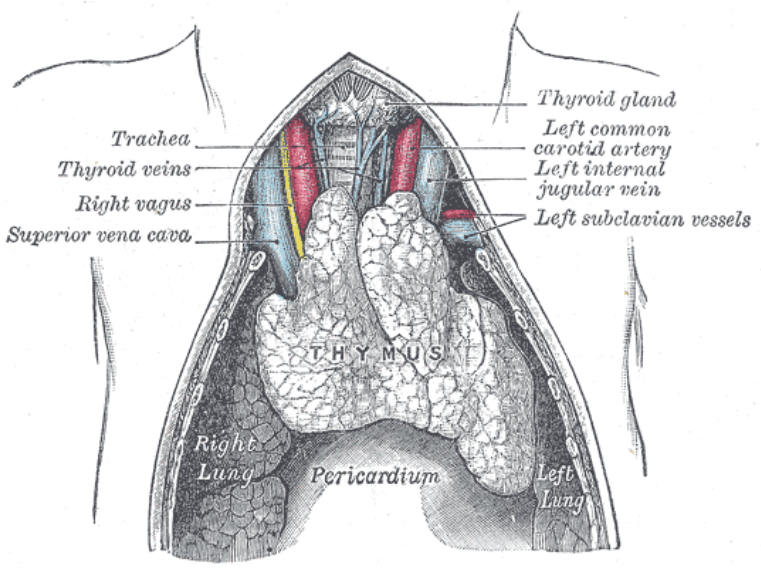
The only vertebrates lacking a spleen are the lampreys and hagfishes. Even in these animals, there is a diffuse layer of haematopoeitic tissue within the gut wall, which has a similar structure to red pulp, and is presumably homologous with the spleen of higher vertebrates.

Chapter 7

Thymus



Thymus



The thymus of a full-term fetus, exposed *in situ*.

Artery	derived from internal mammary artery, superior thyroid artery, and inferior thyroid artery
Nerve	vagus
Lymph	tracheobronchial, parasternal
Precursor	third branchial pouch
MeSH	*Thymus+gland*

Dorlands/Elsevier *Thymus*

The **thymus** is a specialized organ of the immune system. The only known function of the thymus is the production and "education" of T-lymphocytes (T cells), which are critical cells of the adaptive immune system. The thymus is composed of two identical lobes and is located anatomically in the anterior superior mediastinum, in front of the heart and behind the sternum.

Histologically, the thymus can be divided into a central medulla and a peripheral cortex which is surrounded by an outer capsule. The cortex and medulla play different roles in the development of T-cells. Cells in the thymus can be divided into thymic stromal cells and cells of hematopoietic origin (derived from bone marrow resident hematopoietic stem cells). Developing T-cells are referred to as thymocytes and are of hematopoietic origin. Stromal cells include thymic cortical epithelial cells, thymic medullary epithelial cells, and dendritic cells.

The thymus provides an inductive environment for development of T-lymphocytes from hematopoietic progenitor cells. In addition, thymic stromal cells allow for the selection of

a functional and self-tolerant T-cell repertoire. Therefore, one of the most important roles of the thymus is the induction of central tolerance.

The thymus is largest and most active during the neonatal and pre-adolescent periods. By the early teens, the thymus begins to atrophy and thymic stroma is replaced by adipose (fat) tissue. Nevertheless, residual T lymphopoiesis continues throughout adult life.

History

The thymus was known to the Ancient Greeks, and its name comes from the Greek word *θυμός* (thumos), meaning heart, soul, desire, life — possibly because of its location in the chest, near where emotions are subjectively felt; or else the name comes from the herb *thyme* (also in Greek *θυμός*), which became the name for a "warty excrescence", possibly due to its resemblance to a bunch of thyme.

Galen was the first to note that the size of the organ changed over the duration of a person's life.

Due to the large numbers of apoptotic lymphocytes, the thymus was originally dismissed as a "lymphocyte graveyard", without functional importance. The importance of the thymus in the immune system was discovered in 1961 by Jacques Miller, by surgically removing the thymus from three day old mice, and observing the subsequent deficiency in a lymphocyte population, subsequently named T-cells after the organ of their origin. Recently, advances in immunology have allowed the function of the thymus in T-cell maturation to be more fully understood.

Development

Embryology

The two main components of the thymus, the lymphoid thymocytes and the thymic epithelial cells, have distinct developmental origins. The thymic epithelium is the first to develop, and appears in the form of two flask-shape endodermal diverticula, which arise, one on either side, from the third branchial pouch (pharyngeal pouch), and extend lateralward and backward into the surrounding mesoderm and neural crest-derived mesenchyme in front of the ventral aorta.

Here they meet and become joined to one another by connective tissue, but there is never any fusion of the thymus tissue proper. The pharyngeal opening of each diverticulum is soon obliterated, but the neck of the flask persists for some time as a cellular cord. By further proliferation of the cells lining the flask, buds of cells are formed, which become surrounded and isolated by the invading mesoderm. Additional portions of thymus tissue are sometimes developed from the fourth branchial pouches.

During the late stages of the development of the thymic epithelium, hematopoietic bone-marrow precursors migrate into the thymus. Normal thymic development thereafter is

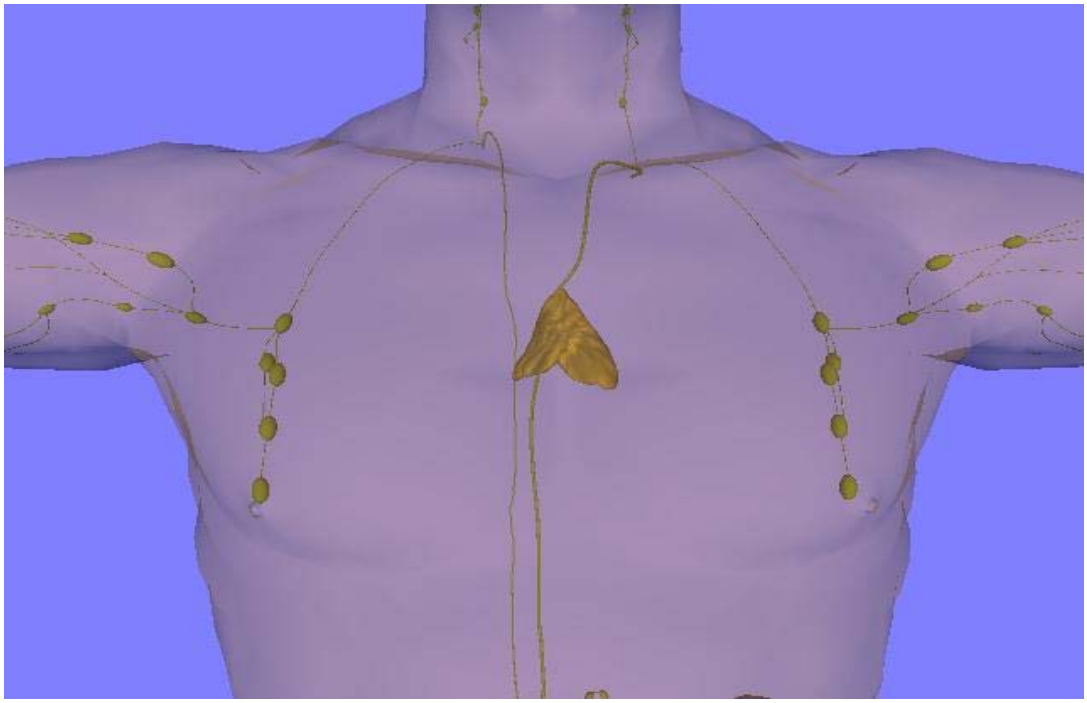
dependant on the interaction between the thymic epithelium and the hematopoietic thymocytes.

Involution

The thymus continues to grow between birth and puberty and then begins to atrophy, a process directed by the high levels of circulating hormones. Proportional to thymic size, thymic activity (T-cell output) is most active before puberty. Upon atrophy, the size and activity are dramatically reduced, and the organ is primarily replaced with fat (a phenomenon known as "organ involution"). The atrophy is due to the increased circulating level of sex hormones, and chemical or physical castration of an adult results in the thymus increasing in size and activity. Patients with the autoimmune disease Myasthenia gravis commonly (70%) are found to have thymic hyperplasia or malignancy. The reason or order of these circumstances has yet to be determined by medical scientists.

Age	**Mass**
birth	about 15 grams;
puberty	about 35 grams
twenty-five years	25 grams
sixty years	less than 15 grams
seventy years	as low as 5 grams

Anatomy

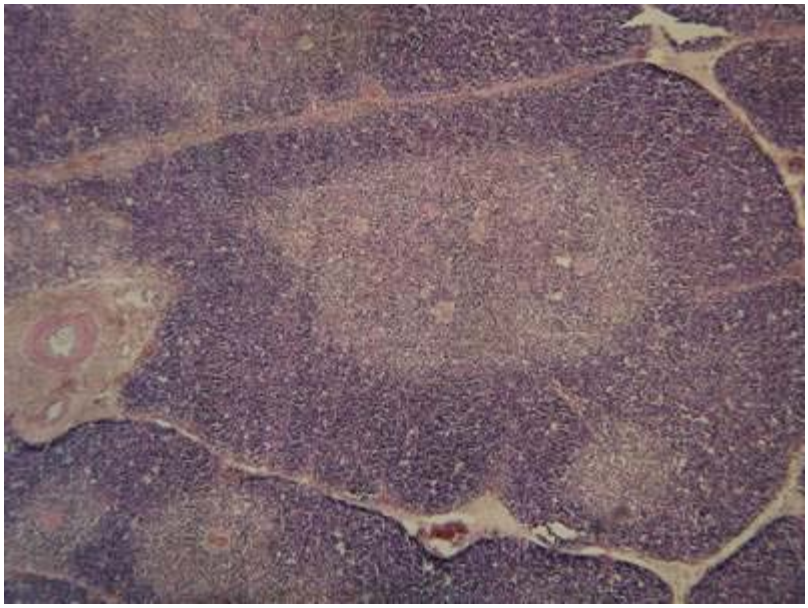


Anterior view of chest showing location and size of adult thymus

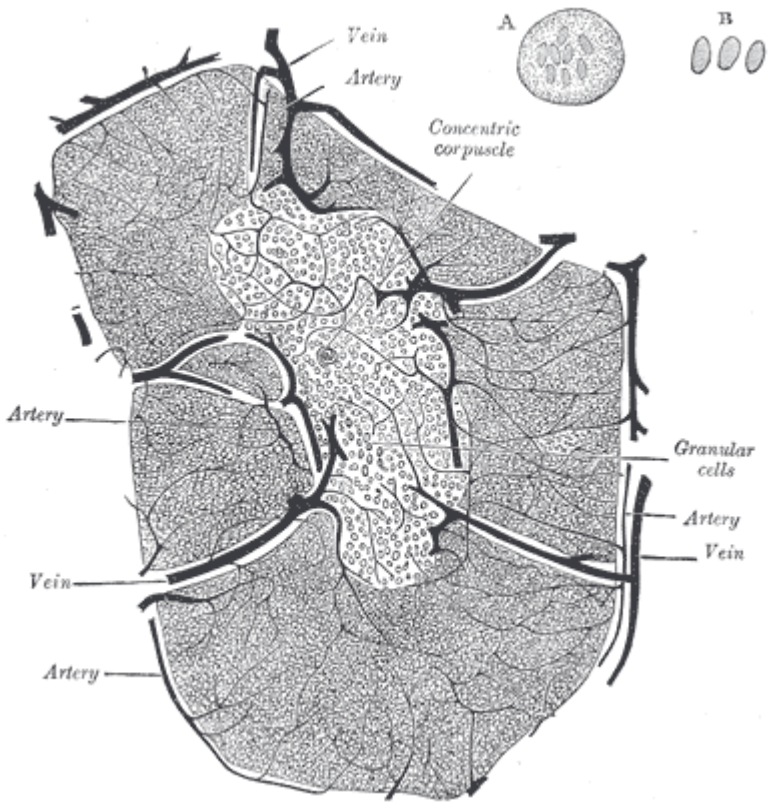
The thymus is of a pinkish-gray color, soft, and lobulated on its surfaces. At birth it is about 5 cm in length, 4 cm in breadth, and about 6 mm in thickness. The organ enlarges during childhood, and atrophies at puberty. Unlike the liver, kidney and heart, for instance, the thymus is at its largest in children. The thymus reaches maximum weight (20 to 37 grams) by the time of puberty. The thymus of older people is scarcely distinguishable from surrounding fatty tissue. As one ages the thymus slowly shrinks, eventually degenerating into tiny islands of fatty tissue. By the age of 75 years, the thymus weighs only 6 grams. In children the thymus is grayish-pink in colour and in adults it is yellow.

The thymus will, if examined when its growth is most active, be found to consist of two lateral lobes placed in close contact along the middle line, situated partly in the thorax, partly in the neck, and extending from the fourth costal cartilage upward, as high as the lower border of the thyroid gland. It is covered by the sternum, and by the origins of the sternohyoidei and sternothyreoidei. Below, it rests upon the pericardium, being separated from the aortic arch and great vessels by a layer of fascia. In the neck, it lies on the front and sides of the trachea, behind the sternohyoidei and sternothyreoidei. The two lobes differ slightly in size and may be united or separated.

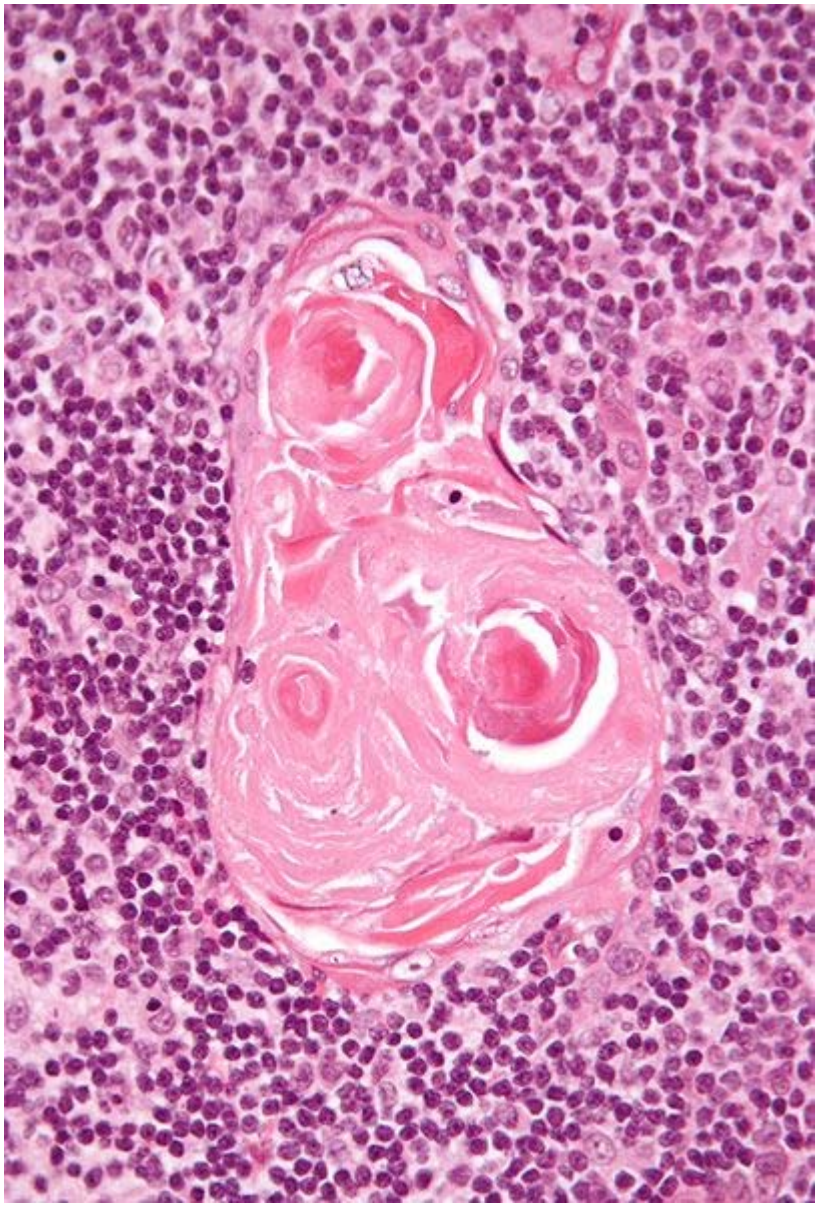
Structure



Histology



Minute structure of thymus



Micrograph showing a thymic corpuscle (Hassall corpuscle), a characteristic histologic feature of the human thymus. H&E stain.

Each lateral lobe is composed of numerous lobules held together by delicate areolar tissue; the entire organ being enclosed in an investing capsule of a similar but denser structure. The primary lobules vary in size from that of a pin's head to that of a small pea, and are made up of a number of small nodules or follicles.

The follicles are irregular in shape and are more or less fused together, especially toward the interior of the organ. Each follicle is from 1 to 2 mm in diameter and consists of a medullary and a cortical portion, and these differ in many essential particulars from each other.

Cortex

The cortical portion is mainly composed of lymphoid cells, supported by a network of finely-branched epithelial reticular cells, which is continuous with a similar network in the medullary portion. This network forms an adventitia to the blood vessels.

The cortex is the location of the earliest events in thymocyte development, where T cell receptor gene rearrangement and positive selection takes place.

Medulla

In the medullary portion, the reticulum is coarser than in the cortex, the lymphoid cells are relatively fewer in number, and there are found peculiar nest-like bodies, the concentric corpuscles of Hassall. These concentric corpuscles are composed of a central mass, consisting of one or more granular cells, and of a capsule formed of epithelioid cells. They are the remains of the epithelial tubes, which grow out from the third branchial pouches of the embryo to form the thymus. Each follicle is surrounded by a vascular plexus, from which vessels pass into the interior, and radiate from the periphery toward the center, forming a second zone just within the margin of the medullary portion. In the center of the medullary portion there are very few vessels, and they are of minute size.

The medulla is the location of the latter events in thymocyte development. Thymocytes that reach the medulla have already successfully undergone T cell receptor gene rearrangement and positive selection, and have been exposed to a limited degree of negative selection. The medulla is specialised to allow thymocytes to undergo additional rounds of negative selection to remove auto-reactive T-cells from the mature repertoire. The gene AIRE is expressed by the thymic medullary epithelium, and drives the transcription of organ-specific genes such as insulin to allow maturing thymocytes to be exposed to a more complex set of self-antigens than is present in the cortex.

Vasculature

The arteries supplying the thymus are derived from the internal mammary, and from the superior thyroid and inferior thyroids.

The veins end in the left brachiocephalic vein (innominate vein), and in the thyroid veins.

The nerves are exceedingly minute; they are derived from the vagi and sympathetic nervous system. Branches from the descendens hypoglossi and phrenic reach the investing capsule, but do not penetrate into the substance of the organ.

Function

In the two thymic lobes, hematopoietic precursors from the bone-marrow, referred to as thymocytes, mature into T-cells. Once mature, T-cells emigrate from the thymus and

constitute the peripheral T-cell repertoire responsible for directing many facets of the adaptive immune system. Loss of the thymus at an early age through genetic mutation (as in DiGeorge Syndrome) results in severe immunodeficiency and a high susceptibility to infection.

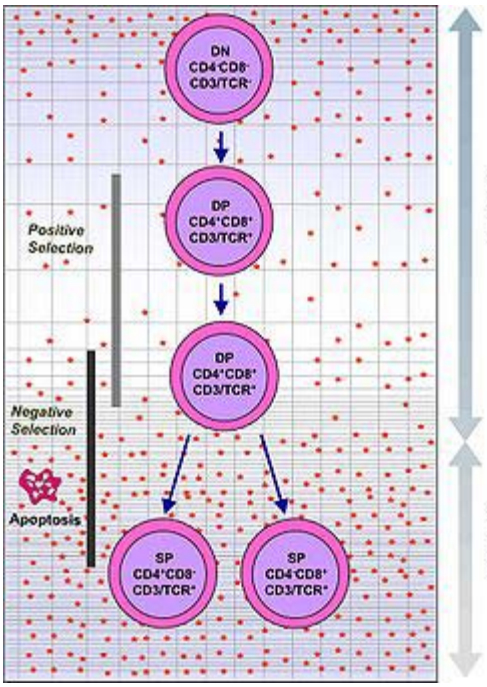
The stock of T-lymphocytes is built up in early life, so the function of the thymus is diminished in adults. It is largely degenerated in elderly adults and is barely identifiable, consisting mostly of fatty tissue, but it continues its endocrine function. Involution of the thymus has been linked to loss of immune function in the elderly, susceptibility to infection and to cancer.

The ability of T-cells to recognize foreign antigens is mediated by the T cell receptor. The T cell receptor undergoes genetic rearrangement during thymocyte maturation, resulting in each T-cell bearing a unique T-cell receptor, specific to a limited set of peptide:MHC combinations. The random nature of the genetic rearrangement results in a requirement of central tolerance mechanisms to remove or inactivate those T cells which bear a T cell receptor with the ability to recognise self-peptides.

Phases of thymocyte maturation

The generation of T-cells expressing distinct T-cell receptors occurs within the thymus, and can be conceptually divided into three phases:

1. A rare population of hematopoietic progenitor cells enter the thymus from the blood, and expands by cell division to generate a large population of immature thymocytes.
2. Immature thymocytes each make distinct T-cell receptors by a process of gene rearrangement. This process is error-prone, and some thymocytes fail to make functional T-cell receptors, whereas other thymocytes make T-cell receptors that are autoreactive.
3. Immature thymocytes undergo a process of selection, based on the specificity of their T-cell receptors. This involves selection of T-cells that are *functional (positive selection)*, and elimination of T-cells that are *autoreactive (negative selection)*.

type:	**functional (positive selection)**	**autoreactive (negative selection)**
location:	cortex	medulla
	In order to be *positively-selected*, thymocytes will have to interact with several cell surface molecules, MHC/HLA, to ensure reactivity and specificity. Positive selection eliminates (apoptosis) weak binding cells and only takes high medium binding cells. (Binding refers to the ability of the T-cell receptors to bind to either MHC class I/II or peptide molecules.)	Negative selection is not 100% complete. Some autoreactive T-cells escape thymic censorship, and are released into the circulation. Additional mechanisms of tolerance active in the periphery exist to silence these cells such as anergy, deletion, and regulatory T cells. If these peripheral tolerance mechanisms also fail, autoimmunity may arise.

Cells that pass both levels of selection are released into the bloodstream to perform vital immune functions.

Disease Associations

Immunodeficiency

As the thymus is the organ of T-cell development, any congenital defect in thymic genesis or a defect in thymocyte development can lead to a profound T cell primary immunodeficiency. Defects that affect both the T cell and B cell lymphocyte lineages result in Severe Combined Immunodeficiency Syndrome (SCID). Acquired T cell deficiencies can also affect thymocyte development in the thymus.

DiGeorge Syndrome

DiGeorge Syndrome is a genetic disorder caused by the deletion of a small section of chromosome 22. This results in a midline congenital defect including thymic aplasia, or congenital deficiency of a thymus. Patients may present with a profound immunodeficiency disease, due to the lack of T cells. No other immune cell lineages are

affected by the congenital absence of the thymus. DiGeorge Syndrome is the most common congenital cause of thymic aplasia in humans. In mice, the nude mouse strain are congenitally thymic deficent. These mice are an important model of primary T cell deficiency.

SCID

Severe combined immunodeficiency syndromes (SCID) are group of rare congenital genetic diseases that result in combined T lymphocyte and B lymphocyte deficencies. These syndromes are cause by defective hematopoietic progenitor cells which are the precursors of both B- and T-cells. This results in a severe reduction in developing thymocytes in the thymus and consequently thymic atrophy. A number of genetic defects can cause SCID, including IL-7 receptor deficiency, common gamma chain deficiency, and Recombination activating gene deficiency.

HIV / AIDS

The HIV virus causes an acquired T-cell immunodeficiency syndrome (AIDS) by specifically killing CD4+ T-cells. Whereas the major effect of the virus is on mature peripheral T-cells, the HIV virus can also infect developing thymocytes in the thymus, most of which express CD4.

Autoimmune Disease

Autoimmune diseases are caused by a hyperactive immune system that instead of attacking foreign pathogens reacts against the host organism (self) causing disease. One of the primary functions of the thymus is to prevent autoimmunity through the process of central tolerance, immunologic tolerance to self antigens.

APECED

Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED) is an extremely rare genetic autoimmune syndrome. However, this disease highlights the importance of the thymus in prevention of autoimmunity. This disease is caused by deficiency of the Autoimmune Regulator (AIRE) gene in the thymus. AIRE allows for the ectopic expression of tissue-specific proteins in the thymus medulla, such as proteins that would normally only be expressed in the eye or pancreas. This expression in the thymus, allows for the deletion of autoreactive thymocytes by exposing them to self-antigens during their development, a mechansism of central tolerance. Patients with APECED develop an autoimmune disease that affects multiple endocrine tissues.

Myasthenia gravis

Myasthenia gravis is an autoimmune disease caused by antibodies that block acetylcholine receptors. Myasthenia gravis is often associated with thymic hypertrophy. Thymectomy may be necessary to treat the disease.

Cancer

Two primary forms of tumours originate in the thymus.

Thymomas

Tumours originating from the thymic epithelial cells are called thymomas, and are found in about 10-15% of patients with myasthenia gravis. Symptoms are sometimes confused with bronchitis or a strong cough because the tumour presses on the recurrent laryngeal nerve. All thymomas are potentially cancerous, but they can vary a great deal. Some grow very slowly. Others grow rapidly and can spread to surrounding tissues. Treatment of thymomas often requires surgery to remove the entire thymus.

Lymphomas

Tumours originating from the thymocytes are called thymic lymphomas. Lymphomas or leukemias of thymocyte origin are classified as Precursor T acute lymphoblastic leukemia/lymphoma (T-ALL).

People with an enlarged thymus, particularly children, were treated with intense radiation in the years before 1950. There is an elevated incidence of thyroid cancer and leukemia in treated individuals.

Thymectomy

Thymectomy is the surgical removal of the thymus. The most common reason for thymectomy in the United States is to gain surgical access to the heart in surgeries to correct congenital heart defects that are performed in the neonatal period. In neonates, but not older children or adults, the relative size of the thymus obstructs surgical access to the heart. Surprisingly, removal of the thymus does not result in a T cell immunodeficiency. This is because sufficient T cells are generated during fetal life prior to birth. These T cells are long-lived and can proliferate by homeostatic proliferation throughout the lifetime of the patient. However, there is evidence of premature immune aging in patients thymectomized during early childhood.

Other indications for thymectomy include the removal of thymomas and the treatment of myastenia gravis. Thymectomy is not indicated for the treatment of primary thymic lymphomas. However, a thymic biopsy may be necessary to make the pathologic diagnosis.

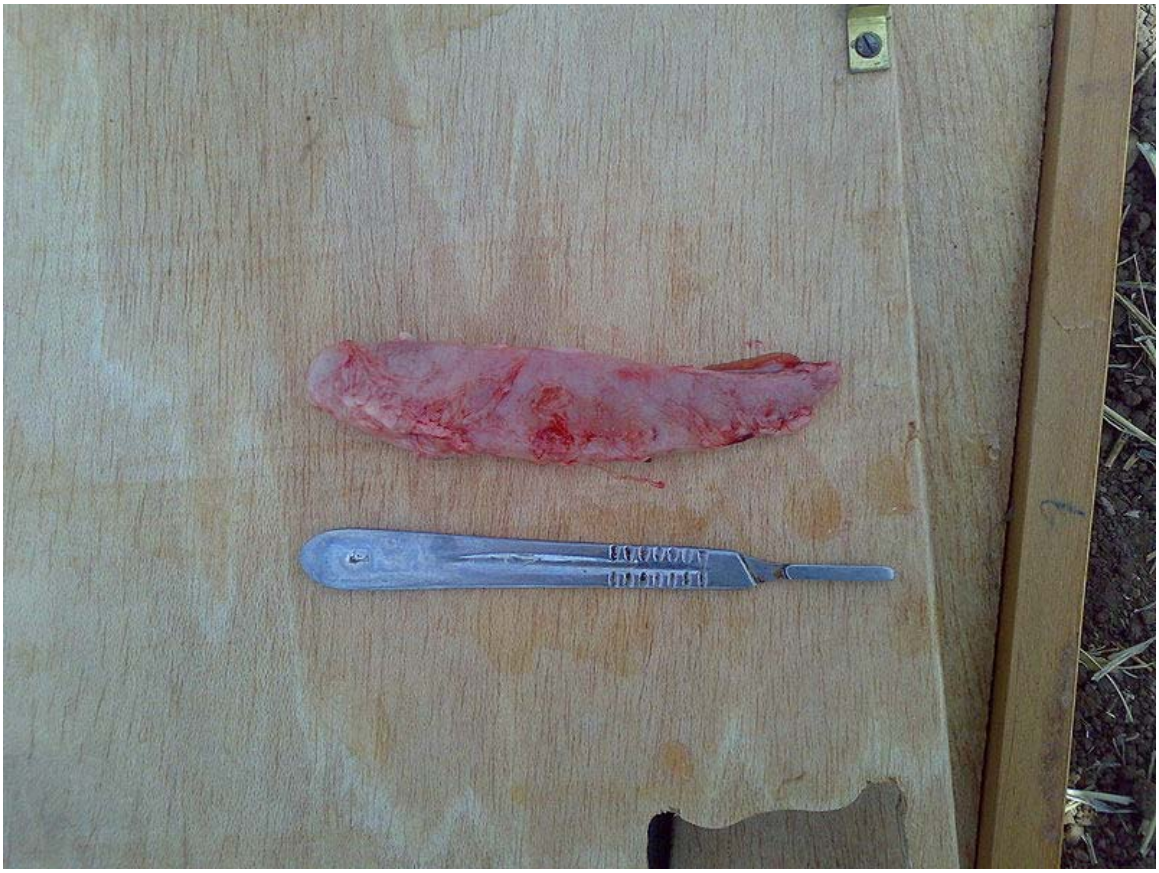
Second thymus

The thymus is also present in most vertebrates, with similar structure and function as the human thymus. Some animals have multiple secondary (smaller) thymi in the neck; this phenomenon has been reported for mice and also occurs in 5 out of 6 human fetuses. As in humans, the Guinea pig's thymus naturally atrophies as the animal reaches adulthood,

but in the athymic hairless guinea pig (which arose from a spontaneous laboratory mutation) possessed no thymic tissue whatsoever, and the organ cavity is replaced with cystic spaces.

Animal thymic tissue sold in a butcher shop or at a meat counter is known as sweetbread.

In animals



A sheep thymus, several times enlarged, in Peste des petits ruminants

Thymus is present in mammals, where it plays the same immunological function as in human beings.

Chapter 8

Gut-Associated Lymphoid Tissue and Lacteal

Gut-associated lymphoid tissue

The digestive tract's immune system is often referred to as **gut-associated lymphoid tissue (GALT)** and works to protect the body from invasion. GALT is an example of mucosa-associated lymphoid tissue.

Function

The digestive tract is an important component of the body's immune system. In fact, the intestine possesses the largest mass of lymphoid tissue in the human body. The GALT is made up of several types of lymphoid tissue that store immune cells, such as T and B lymphocytes, that carry out attacks and defend against pathogens.

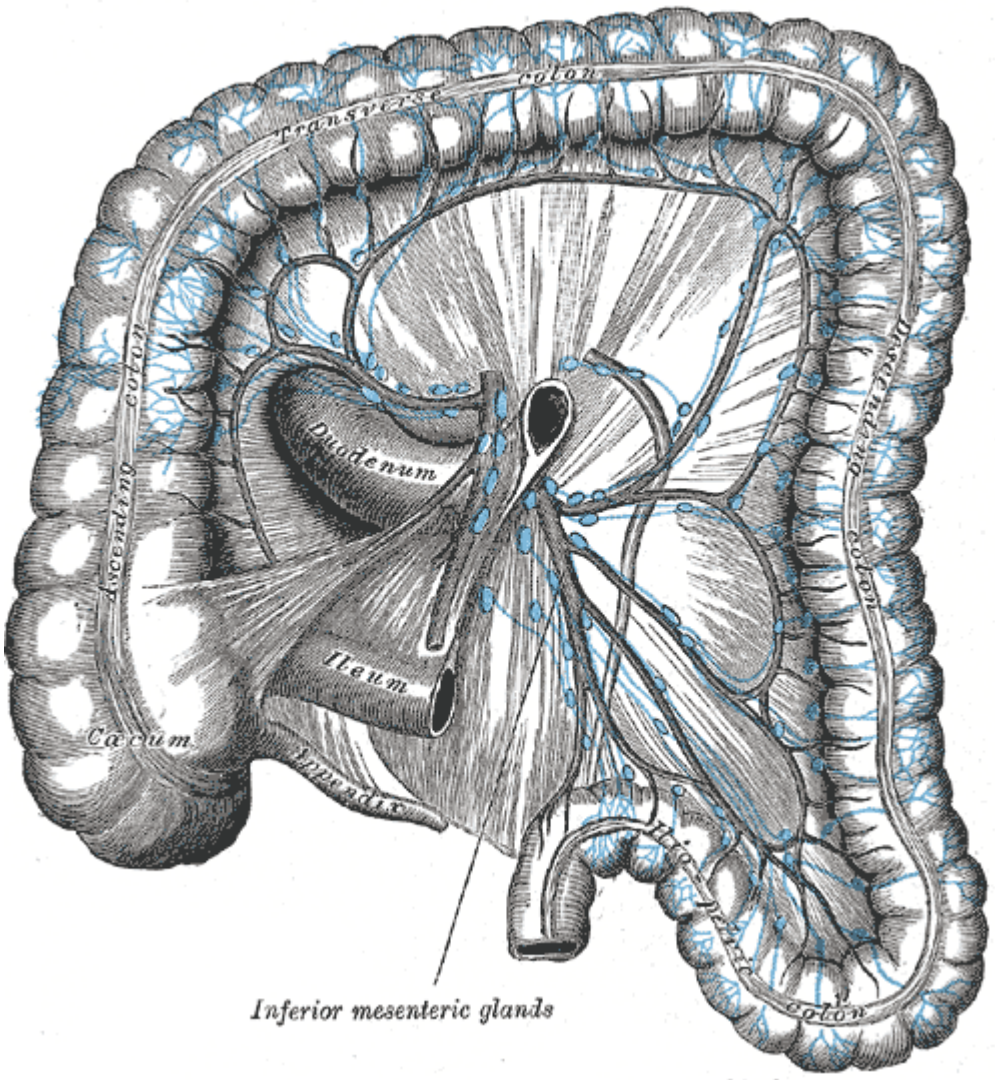
New research indicates that GALT may continue to be a major site of HIV activity, even if drug treatment has reduced HIV count in the peripheral blood.

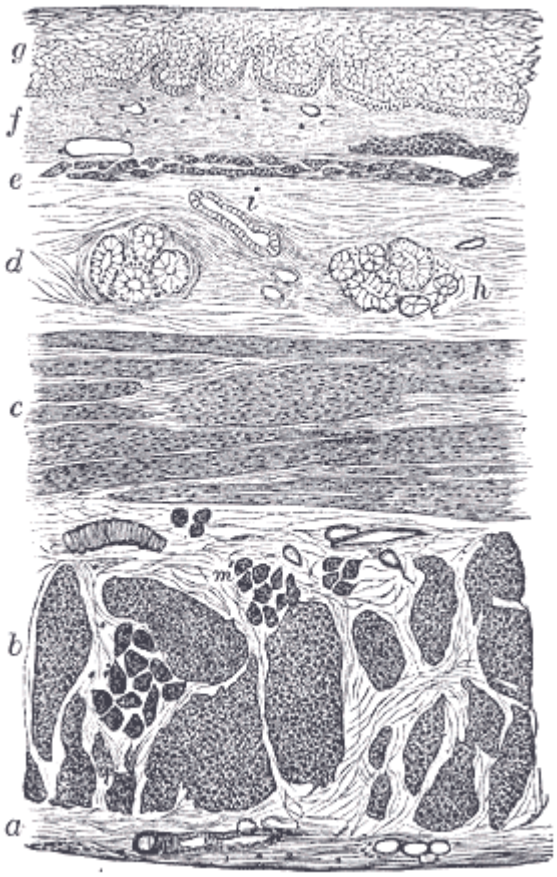
Components

Lymphoid tissue in the gut comprises the following:

- Tonsils (Waldeyer's ring)
- Adenoids (Pharyngeal tonsils)
- Peyer's patches
- Lymphoid aggregates in the appendix and large intestine
- Lymphoid tissue accumulating with age in the stomach
- Small lymphoid aggregates in the esophagus
- Diffusely distributed lymphoid cells and plasma cells in the lamina propria of the gut

Additional images

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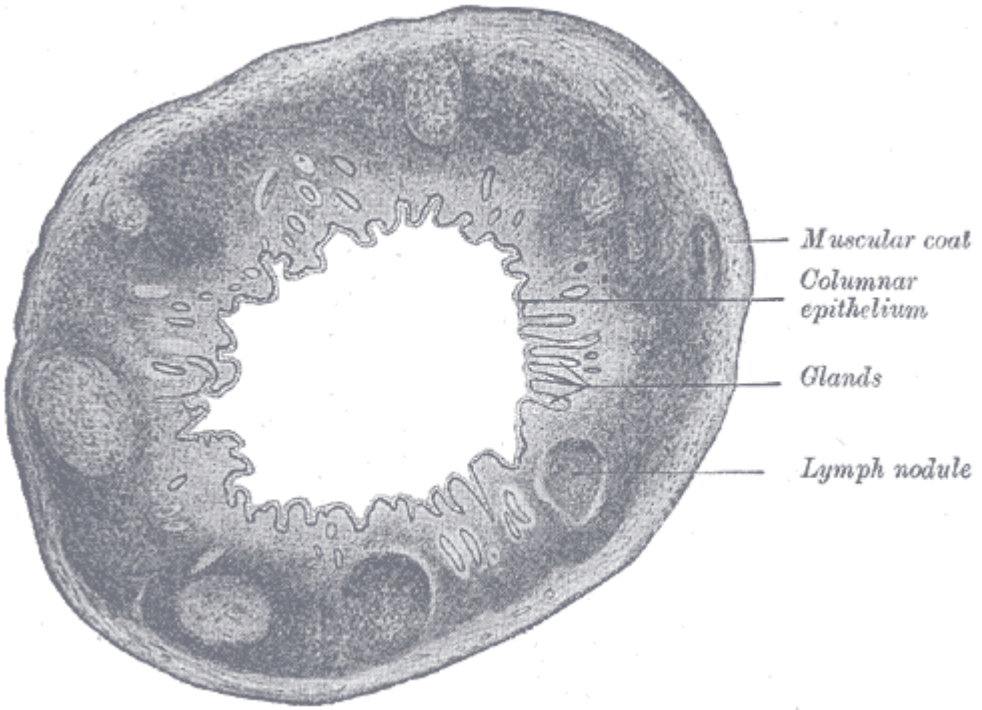
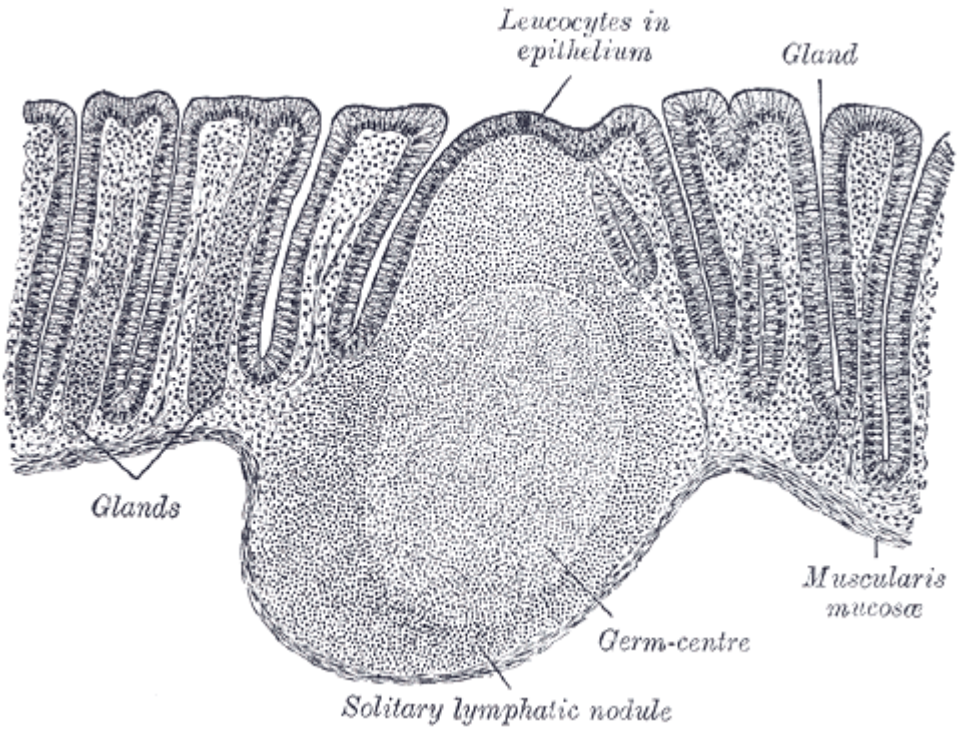
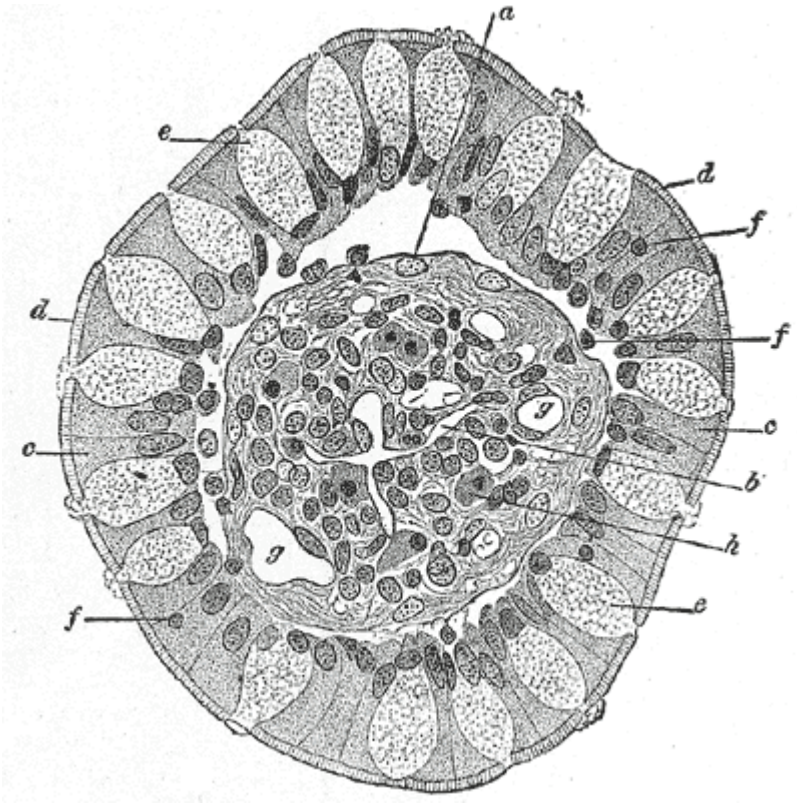

Muscular coat
Columnar epithelium
Glands
Lymph nodule


Leucocytes in epithelium
Gland
Glands
Muscularis mucosæ
Germ-centre
Solitary lymphatic nodule

Lacteal

Lacteal



Transverse section of a villus, from the human intestine. X 350.

a. Basement membrane, here somewhat shrunken away from the epithelium.

b. Lacteal.

c. Columnar epithelium.

d. Its striated border.

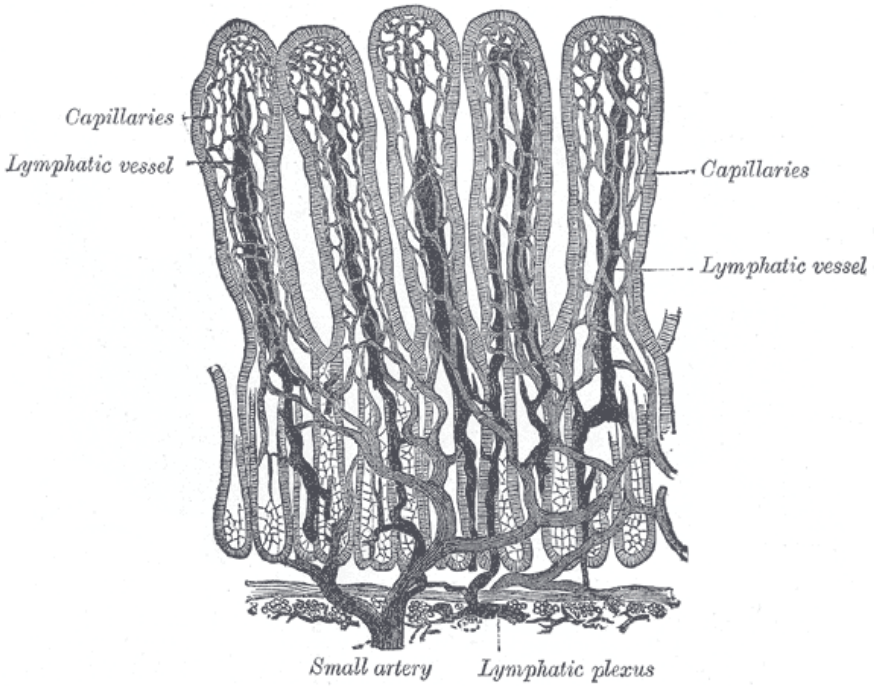
e. Goblet cells.

f. Leucocytes in epithelium.

f'. Leucocytes below epithelium.

g. Bloodvessels.

h. Muscle cells cut across.



Villi of small intestine, showing bloodvessels and lymphatic vessels.

A **lacteal** is a lymphatic capillary that absorbs dietary fats in the villi of the small intestine.

The combination of fat and lymph in the lacteals is milky in appearance and called chyle. Individual lacteals merge to form larger lymphatic vessels that transport the fats to the thoracic duct which empties into the left subclavian vein found under the collar bone.

At this point, the fats are in the bloodstream in the form of chylomicrons. Once in the blood, chylomicrons are subject to delipidation by lipoprotein lipase. Eventually, enough lipid has been lost and additional apolipoproteins gained, that the resulting particle (now referred to as a chylomicron remnant) can be taken up by the liver. From the liver, the fat released from chylomicron remnants can be re-exported to the blood as the triglyceride component of very low density lipoprotein (VLDL). VLDL, also subject to delipidation by vascular lipoprotein lipase, delivers fats to tissues throughout the body and, in particular, the released fatty acids can be stored in adipose cells as triglycerides. As triglycerides are lost from VLDL the lipoprotein particle becomes smaller and denser (since protein is denser than lipid) and ultimately becomes low density lipoprotein (LDL). A lot has been written about LDL because it is thought to be atherogenic.

Note that in contrast to any other route of absorption from the small intestine, the lymphatic system avoids first pass metabolism.

Chapter 9

Lymph Capillary, Lymph Node Biopsy and Lymphangiogenesis

Lymph capillary

Lymph capillary

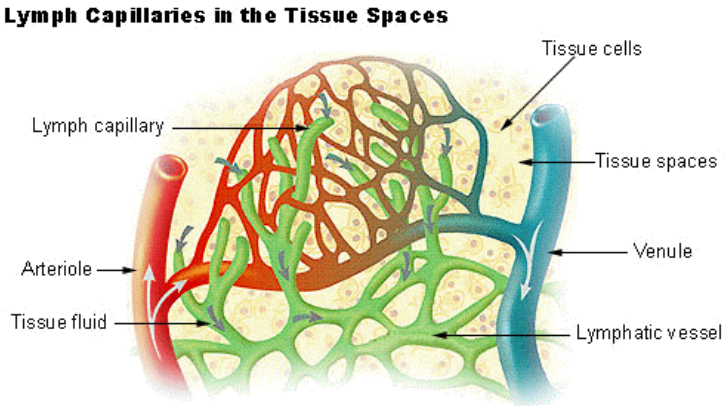


Diagram showing the formation of lymph from interstitial fluid (labeled here as "Tissue fluid"). Note: how the tissue fluid is entering the blind ends of lymph capillaries (indicated by deep green arrows)

Latin *vas lymphocapillare*

Lymph capillaries or **lymphatic capillaries** are tiny thin-walled vessels that are closed at one end and are located in the spaces between cells throughout the body, except in the central nervous system, and in non-vascular tissues. The main purpose of these vessels is to drain excess tissue fluids from around the cell ready to be filtered and returned to the venous circulation. This tissue fluid upon entering the lumen (elongated cavity of a tubular structure) is known as the lymph.

Lymphatic capillaries are slightly larger in diameter than blood capillaries and have a unique structure that permits interstitial fluid to flow into them but not out. The ends of

endothelial cells that make up the wall of a lymphatic capillary overlap. When pressure is greater in the interstitial fluid than in lymph, the cells separate slightly, like the opening of a one-way swinging door, and interstitial fluid enters the lymphatic capillary. When pressure is greater inside the lymphatic capillary, the cells adhere more closely, and lymph cannot escape back into interstitial fluid. Attached to the lymphatic capillaries are anchoring filaments, which contain elastic fibers. They extend out from the lymphatic capillary, attaching lymphatic endothelial cells to surrounding tissues. Lymph capillaries have a greater oncotic pressure, which is due to the greater concentration of plasma proteins in the lymph.

The lymphatic capillary becomes the afferent lymphatic vessel and carries the lymph into a lymph node.

When excess interstitial fluid accumulates and causes tissue swelling, the anchoring filaments are pulled, making the openings between cells even larger so that more fluid can flow into the lymphatic capillary.

Lymph node biopsy

Lymph node biopsy is a test in which a lymph node or a piece of a lymph node is removed for examination under a microscope.

The lymphatic system is made up of several lymph nodes connected by lymph vessels. The nodes produce white blood cells (lymphocytes) that fight infections. When an infection is present, the lymph nodes swell, produce more white blood cells, and attempt to trap the organisms that are causing the infection. The lymph nodes also try to trap cancer cells.

How the test is performed

The test is done in an operating room in a hospital, or at an outpatient surgical facility. There are two ways the sample may be obtained:

- Needle biopsy
- Open (excisional) biopsy

Needle biopsy

A needle biopsy involves inserting a needle into a node to obtain the sample.

The patient lies on the examination table; the biopsy site is cleansed; and a local anesthetic is injected. The biopsy needle is then inserted into the node. A sample is removed, pressure is applied to the site to stop the bleeding, and a bandage is applied.

Open biopsy

An open biopsy consists of surgically removing all or part of a node.

The patient lies on the examination table and is given a sedative. The skin over the biopsy site is cleansed, and a local anesthetic is injected (occasionally, a general anesthetic is given). A small incision is made, and the lymph node or part of the node is removed. The incision is then closed with stitches and bandaged.

The sample is then sent to pathology.

With this test there is a small chance of infection or bleeding. Additionally, there is a moderate risk of nerve injury, localized paralysis, or numbness when the biopsy is performed on a lymph node close to nerves.

Test results

The test is used to help determine the cause of lymph node enlargement (swollen glands or lymphadenitis). It may also determine whether tumors in the lymph node are cancerous or noncancerous. Enlarged lymph nodes may be caused by a number of conditions, ranging from very mild infections to serious malignancies. Benign conditions can often be distinguished from cancerous and infectious processes by microscopic examination. The pathologist may also perform additional tests on the lymph node tissue to assist in making a diagnosis.

Some of the conditions where abnormal values are obtained are:

- Hodgkin's lymphoma
- Non-Hodgkin's lymphoma
- Sarcoidosis
- tuberculous cervical lymphadenitis (scrofula)

Lymphangiogenesis

Lymphangiogenesis is the formation of lymphatic vessels from pre-existing lymphatic vessels, in a method believed to be similar to blood vessel development or angiogenesis.

Lymphangiogenesis plays an important physiological role in homeostasis, metabolism and immunity. Lymphatic vessel formation has also been implicated in a number of pathological conditions including neoplasm metastasis, oedema, rheumatoid arthritis, psoriasis and impaired wound healing.

The role of the lymphatic system in these diseases has received renewed interest largely due to the recent discovery of specific lymphatic markers such as Podoplanin, LYVE-1, PROX-1, desmoplakin, VEGF-C, VEGF-D and their receptor VEGFR-3. These specific markers have enabled new insights into functional and molecular lymphatic biology.

The LECs（lymphatic endothelial cells） which has closely relationship with lymphangiogenesis are terminally differentiated cells distinct from blood vascular endothelial cells.

There are several lymphangiogenesis inducer such as hyaluronic acid and Ephrin-B2.

Useful resources

Lymphangiogenesis and disease

- Tumor-induced lymphangiogenesis:
- Lymphangiogenesis and prosthetic joint failure:

Chapter 10

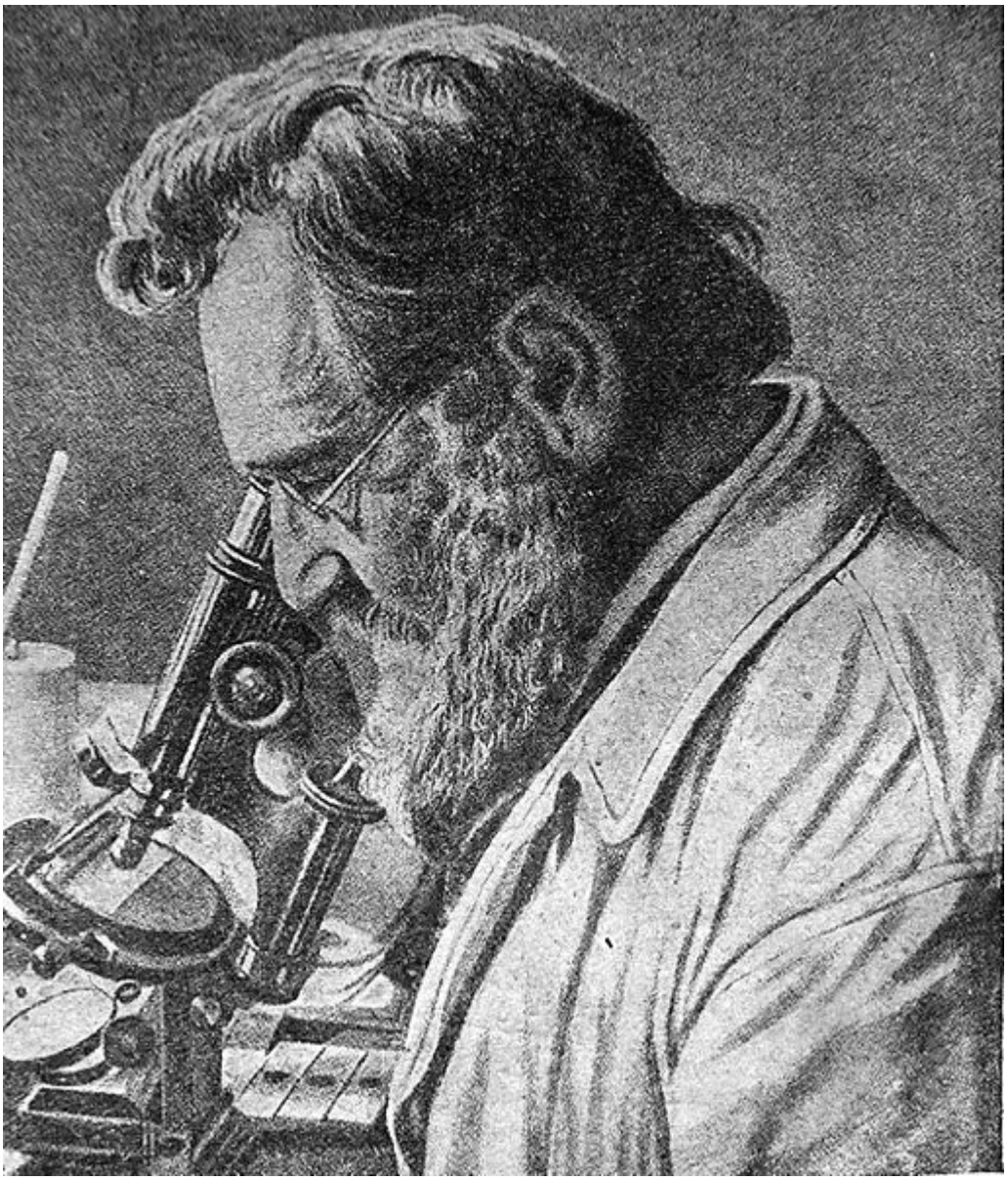
Lymphopoiesis

Lymphopoiesis Glossary
• **antigen** any molecule that can provoke an immune defense
• **B cells** lymphocytes that ultimately produce antibodies
• **bone marrow** the center of bones capable of producing all red and white blood cells in the adult
• **cortex** the outer portion of any organ
• **cytoplasm** the portion of a cell between the nucleus and the membrane
• **differentiation** permanent changes to a cell developing over time and with cell division
• **granules** grains found in many white blood cells, composed of defensive chemicals
• **hematopoietic** that which gives rises to any blood cell type
• **lineage** a type of cell and its descendants by division and differentiation
• **lymphocytes** a special 'lineage' of WBC
• **macrophages** myeloid descendants (some may be lymphoid) with 'eating' abilities, also cooperate with lymphocytes
• **myeloid** ancestors of WBCs with granules and also of macrophages
• **T Cells** "management" lymphocytes for immunity
• **(WBC) White Blood Cell** in contrast to the much more common Red Blood Cell; responsible for defense

Lymphopoiesis (lĭm'fō-poi-ē'sĭs) refers to the generation of lymphocytes, one of the five different types of white blood cells (WBC), and is also more formally called lymphoid hematopoiesis.

The name Lymphopoiesis

Lymphocytes are considered to be of the lymphoid lineage as opposed to other lineages of blood cells such as the myeloid lineage and the erythroid lineage.



Immunology pioneer Elie Metchnikoff

Nomenclature, the problem of naming things properly, is not trivial in this case because lymphocytes - which are certainly found in blood and originate along with blood cells in

the bone marrow of the adult - are almost by definition also strongly associated with lymph fluid and a separate connective system, the lymphatic system, which is assuredly not the blood system. Connected to, parallel to and interacting with and feeding into the blood system, yes, but easily distinguished apart by any scientific observer.

Lymphopoiesis is now used interchangeably with the term "lymphocytopoiesis" - the making of lymphocytes - but other sources may distinguish between the two, stating that "lymphopoiesis" additionally refers to creating lymphatic tissue, while "lymphocytopoiesis" refers only to the creation of cells in that tissue. It is rare now for lymphopoiesis to refer to the creation of lymphatic tissues.

Myelopoiesis refers to 'generation of cells of the myeloid lineage' and erythropoiesis refers to 'generation of cells of the erythroid lineage' etc., so parallel usage has evolved in which lymphopoiesis refers to 'generation of cells of the lymphoid lineage'.

Observations on research going back well over 100 years had elucidated the two great classes of WBC - Myeloid and Lymphoid - and great advances in medicine and science have resulted from these studies. It was only natural to ask where these two great classes of cells arose, and after much work two cell types with some strong stem cell properties were isolated and defined - CMP, the common myeloid progenitor and CLP, the common lymphoid progenitor for mice. But science is an additive game and it was eventually found these progenitors were not unique, and further that the two great families of Myeloid and Lymphoid were not disjoint, but rather two partially interwoven family trees. This is more than just nomenclature, it is new science that provides challenges of complexity yet offers new vistas of bio-science and the promise of early enhancement of private and public health issues. And it gives insight into the nature of redundancy and overlap in the immune system and hints how to use this to advantage.

The purpose of Lymphopoiesis

The complete loss of or loss of function of any WBC cell type is a serious health matter, but lymphopoiesis is absolutely necessary for life. Mature lymphocytes are a critical part of the immune system that (with the exception of memory B and T cells) have short lives measured in days or weeks and must be continuously generated throughout life by cell division and differentiation from cells such as common lymphoid progenitors (CLPs) in mice. Were this system to fail, the body would be largely undefended from infection.

The set comprising CLP cells and similar progenitors are themselves descendants of the pluripotential hemopoietic stem cell (pHSC) which is capable of generating all of the cell types of the complete blood cell system. Despite their remarkable ability to generate the complete suite of lymphocytes, most progenitors are not true stem cells, however, and must be continually renewed by differentiation from the pHSC stem cell.

Many progenitor cells are also referred to as *transit cells*,, sometimes also called *transit amplifying cells*, the meaning of this term being that the transit cell may found a new sub-lineage but the number of resultant cells is strictly limited (although possibly very large,

even trillions yet finite) and the lineage is terminated by cells that die off (by apoptosis) or remain as cells that can no longer divide. Examples of such cells are CFUs (Colony-forming units - referred to as such because of their ability to form colonies in vitro in artificial media) such as CFU-T.

In mice, transplantation of a single pHSC cell can reconstitute a sub-lethally irradiated host (i.e. a mouse that has been irradiated so that all leukocytes are killed) with all these lineages of cells, including all types of lymphocytes via CLPs. This has been known for more than 40 years.

Lymphopoiesis continues throughout life and so progenitor cells and their parent stem cells must always be present.

Overview of Lymphopoiesis

In the case of mammals such as man lymphopoiesis begins with limited passive provision by the mother of lymphocytes and substantial immunoglobulin G that cross the placenta and enter the fetus to provide some protection against pathogens, and also leukocytes that come from breast milk and enter circulation via the digestive tract.

However early in gestation the developing embryo has begun its own lymphopoiesis from the fetal liver. Lymphopoiesis also arises from the yolk sac. This is in contrast to the adult where all lymphocytes originate in the bone marrow.

There are four major types of lymphocytes, many sub-types, and hundreds or thousands of lymphocyte cell types that have been identified by scientists. All are generated by normal or abnormal lymphopoiesis except for certain artificial strains created in the laboratory by development from existing strains. Although lymphocytes are usually considered mature (as seen in blood tests) they are certainly not inert but can and do get around the body to anywhere there is a need; and when such need arises, new rounds of 'downstream' lymphopoiesis such as cell multiplication and differentiation may arise, coupled with intense mitotic and metabolic activity.

This is hardly a simple topic. In his 1976 text *Immunology, Aging and Cancer* immunologist and Nobel Prize winner Sir Frank Macfarlane Burnet speculated that the immune system might one day be found to be as complex as the nervous system. As the production of lymphocytes is so close to the central role of the immune response it is wise to approach the study of it with some humility in the face of the task, although there are general principles that help in understanding.

The process of Lymphopoiesis

Lymphopoiesis Acronyms
• **B-NK** Progenitor for B and NK
• **CB** Cord blood
• **CFU** Colony-forming Unit
• **CLP** Common Lymphoid Progenitor
• **CMP** Common Myeloid Progenitor
• **DC** Dendritic Cell (Myeloid or Lymphoid)
• **ELP** Early Lymphoid Progenitors
• **ETP** the most primitive cells in the thymus are the Early Thymocyte Progenitors
• **G-CSF** Granulocyte Colony Stimulating Factor
• **GM-CSF** Granulocyte Macrophage Colony Stimulating Factor
• **GMP** Granulocyte Macrophage Progenitor;
• **HSC** pluripotential Hemopoietic Stem Cell
• **MDC** combined Macrophage and DC progenitor potential
• **MEP** megakaryocytic and erythroid progenitor
• **MLP** Multi-lymphoid Progenitor potential, any progenitor minimally able to give rise to B cells, T cells and NK cells
• **MPP** Multipotent Progenitor
• **Notch** Notch signaling pathway re T Cell commitment from progenitors

Lymphopoiesis can be viewed in a *mathematical* sense as a recursive process of cell division and also as a process of differentiation, measured by changes to the properties of cells.

- Given that lymphocytes arise from specific types of limited stem cells - which we can call P (for Progenitor) cells - such cells can divide in several ways. These are general principles of limited stem cells.

Considering the P as the ‘mother’ cell, but not a true stem cell, it may divide into two new cells, which are themselves identical, but differ to some degree from the mother. Or the mother cell P may divide unequally into two new daughter cells both of which differ from each other and also from the mother.

Any daughter cell will usually have new specialized abilities and if it is able to divide it will form a new sub-lineage. The difference of a daughter cell from the mother may be great, but it could also be much less, even subtle. What the P mother cell does *not* do is divide into two new P mother cells or a mother and a daughter; this is a matter of observation as such limited progenitor cells are known to not self-renew.

- There is a sort of exception when daughter cells at some level of the lineage may divide several times to form more seemingly identical cells, but then further differentiation and division will inevitably occur, until a final stage is reached in which no further division can occur and the cell type lineage is finally mature. An example of maturity is a plasma cell, from the B cell lineage, which produces copious antibody, but cannot divide and eventually dies after a few days or weeks.

- The progenitor CLP of the mouse or the progenitor MLP of the human differentiates into lymphocytes by first becoming a lymphoblast (Medical Immunology, p. 10). It then divides several more times to become a prolymphocyte that has specific cell-surface markers unique to either a (1) T cell or (2) B cell. The progenitor can also differentiate into (3) natural killer cells (NK) and (4) dendritic cells.

- T Cells, B Cells and NK Cells are unique to the lymphocyte family, but dendritic cells are not. Dendritic cells of identical appearance but different markers are spread throughout the body, and come from either lymphoid and myeloid lineages, but these cells may have somewhat different tasks and may take up lodging preferentially in different locations. (Revise in light of new research) This is now an open question; also, the different dendritic cell lineages may have different 'tasks' and stay in different 'locations.'

T and B lymphocytes are indistinguishable histologically（that is, under a light microscope they cannot be told apart.）Indeed, the inactive B and T cells are so featureless with few cytoplasmic organelles and mostly inactive chromatin that until the 1960s textbooks could describe these cells, now the central focus of immunology, as having no known function!!

However T and B lymphocytes are very distinct cell lineages and they 'grow up' in different places in the body. They perform quite different (although co-operative) functions in the body. No evidence has ever been found that T and B cells can ever interconvert. T and B cells are biochemically distinct and this is reflected in the differing markers and receptors they possess on their cell surfaces. This seems to be true in all vertebrates, although there are many differences in the details between the species.

- Regardless of whether the CLP (mouse) or MLP or a small closely related set of progenitor cells take credit for generating the profusion of lymphocytes, it remains an interesting observation that the same lymphoid progenitors can still opt to generate some cells that are clearly identifiably myeloid.

Lymphopoiesis for T cells

T cells are formed in bone marrow then migrate to the cortex of the thymus to undergo maturation in an antigen-free environment for about one week where a mere 2-4% of the T cells succeed. The remaining 96-98% of T cells die by apoptosis and are phagocytosed by macrophages in the thymus. So many thymocytes (T cells) die during the maturation process because there is intensive screening to make sure each thymocyte has the ability to recognize self-peptide:self-MHC complex and for self tolerance. The apoptosed thymocyte dies a willing and noble death and it is quickly recycled.

Upon maturity, there are several forms of thymocytes including

- T-helper (needed for activation of other cells such as B cells and macrophages),
- T-cytotoxic (which kill virally-infected cells),
- T-memory (T cells that remember antigens previously encountered), and
- T-suppressor cells (which moderate the immune response of other leukocytes). Also called T-regulatory Cell, or just 'Treg(s)', to be cool.

When T-Cells become activated they undergo a further series of developments. A small, resting T lymphocyte rapidly undergoes blastogenic transformation into a large lymphocyte (13–15μm). This large lymphocyte (known in this context as a lymphoblast) then divides several times to produce an expanded population of medium (9–12μm) and small lymphocytes (5–8μm) with the same antigenic specificity. Final activated and differentiated T lymphocytes are once again morphologically indistinguishable from a small, resting lymphocyte. Thus the following developmental states may be noticed in sequence in blood tests:

1. Prolymphocyte
2. Large lymphocyte
3. Small lymphocyte

Basic Map of T Cell lymphopoiesis

This basic map of T Cell formation, in sequence, is simplified and is akin to textbook descriptions, and may not reflect latest research. (Medical Immunology, p.119)

In the thymus

- MLP
- ETP
- DN1
- (B; Mφ)
- DN2
    - (DC; NK)
- DN3
    - (γδ)

- DN4
- DP
    - (TNK; CD4; CD8; Treg)

In the Periphery

- (Th1; Th2)

More details on T Lymphopoiesis

Unlike other lymphoid lineages, T cell development occurs almost exclusively in the thymus. T-lymphopoiesis does not occur automatically but requires signals generated from the thymic stromal cells. The process has an astonishingly complex beauty to it. Several stages at which specific regulators and growth factors are required for T cell development to proceed have been defined. Interestingly, later in T cell development and its maturation these same regulatory factors again are used to influence T cell specialization.

T cells are unique among the lymphocyte populations in their ability to further specialize as mature cells and become yet more mature. And T Cells come in many flavors, for example: the conventional TcRαβ T cells; the so-called unconventional TcRγδ T cells; NKT cells; and T regulatory cells (Treg). Details regarding the developmental and life cycle of the unconventional T cells are less well-described compared to the conventional T cells.

Stages of T cell maturation

Stage One: Thymic Migration

Multipotent lymphoid progenitors (MLP) enter the T cell pathway as they immigrate to the thymus. The most primitive cells in the thymus are the early thymocyte progenitors (ETP), which retain all lymphoid and myeloid potential but exist only transiently, rapidly differentiating into T and NK lineages. (Medical Immunology, p. 118)

Stage Two: Proliferative Expansion and T Lineage Commitment

Final commitment to the T cell lineage occurs within the thymic microenvironment, the microscopic structures of the thymus where T cells are nurtured. The most primitive T cells retain pluripotential ability and can differentiate into cells of the myeloid or lymphoid lineages (B cells, DC, T cells, or NK cells).

More differentiated double negative T cells (DN2 cells) have more limited potentiality but are not yet fully restricted to the T cell lineage (they can still develop into DC, T cells, or NK cells). Later on, they are fully committed to the T cell lineage- when thymoctyes expressing Notch1 receptors engage thymic stromal cells expressing Notch1 ligands, the thymocytes become finally committed to the T-cell lineage.

With the commitment to the T cell lineage, begins a very complex process known as TcR gene rearrangement. This creates an enormous diversity of T cells bearing antigen receptors. Afterward some T cells leave the thymus to migrate to the skin and mucosae.

Stage Three: β-Selection

Stage Four: T Cell Receptors Selection

Only 2% to 3% of the differentiating thymocytes, those that express TcR capable of interaction with MHC molecules, but tolerant to self-peptides, survive the Stage Four selection process.

Stage Five: Continuing Differentiation in the Periphery

It was previously believed that the human thymus remained active as the site of T cell differentiation only until early adulthood and that later in adult life the thymus atrophies, perhaps even vanishing. Recent reports indicate that the human thymus is active throughout adult life. Thus several factors may contribute to the supply of T cells in adult life: generation in the thymus, extra-thymic differentiation, and the fact that memory T cells are long-lived and survive for decades.

Even more details

- Unconventional T cells

The thymus also gives rise to the so-called 'unconventional T cells' such at γδ T cells, Natural Killer T cells (NKT) and regulatory T cells (Treg).

- γδ T cells

γδT cells represent only 1% to 5% of the circulating T cells, but are abundant in the mucosal immune system and the skin, where they represent the dominant T cell population. These 'non-MHC restricted T cells' are involved in specific primary immune responses, tumor surveillance, immune regulation and wound healing.

Several differences between αβ and γδ T cell development have been described. They emigrate from the thymus in "waves" of clonal populations, which home to discrete tissues. For example, one kind is found in the peripheral blood while another predominates in the intestinal tract.

- Natural Killer T cells

Human NKT cells are a unique population and are thought to play an important role in tumor immunity and immunoregulation.

- T Regulatory cells

"Tregs" are considered as naturally occurring regulatory T cells. Tregs comprised about 5% of the circulating CD4+ T cells. These cells are thought to possess important an autoimmunity property by regulating 'autoreactive' T cells in the periphery. (Medical Immunology, p. 117-122)

Lymphopoiesis for B cells

B cells are formed and mature in bone marrow (and spleen).

It is a good mnemonic aide that B cells are formed in the bone marrow, but it is a mere coincidence since B cells were first studied in the chicken's bursa of Fabricius and it is from this bursa that B cells get their name.

These B cells then leave the bone marrow and migrate to peripheral lymphoid tissues, such as a lymph node. Once in a secondary lymphoid organ the B cell can be introduced to an antigen that it is able to recognize.

Through this antigen recognition and other cell interactions the B cell becomes activated and then divides and differentiates to become a plasma cell. The plasma cell, a B cell end product, is a very active antibody-secreting cell that helps protect the body by attacking and binding to antigen.

Even after many decades of research, some controversy remains as to where B cells mature and 'complete their education', with the possibility remaining that the site may also partially be peri-intestinal lymphoid tissues.

B lymphopoiesis occurs exclusively in the bone marrow and B lymphocytes are made continuously throughout life there in a 'microenvironment' composed of stromal cells, extracellular matrix, cytokines and growth factors, which are critical for proliferation, differentiation, and survival of early lymphocyte and B-lineage precursors.

The relative proportion of precursor B cells in the bone marrow remains rather constant throughout the life span of an organism. There are stages such as Pre-B-I cells (5% to 10% of the total); Pre-B-II cells (60% to 70%) while the remaining 20% to 25% are immature B cells. Most textbooks say that B Cells mature in the bone marrow but, generally, immature B cells migrate to the spleen for 'higher education' of some sort where they go through transitional stages before final maturation.(Medical Immunology, p. 136)

B lymphocytes are identified by the presence of soluble immunoglobulin G (IgG). This is the most common protective immunoglobulin in the adult body. After antigenic stimulation, B cells differentiate into plasma cells that secrete large quantities of soluble IgG. This is the final stage of B lymphopoiesis but it is the clincher because the plasma cells must either issue antibody close to a source of infection, or disseminate it in the blood to fight an infection at a distance or in an inaccessible part of the body.

Basic map of B cell lymphopoiesis

A generally regarded valid map of B cell lymphopoiesis is as follows in sequence, in two parts with the first being in the bone marrow and the second in the spleen:. The development process in the bone marrow occurs in Germinal Centers.

In the bone marrow

- Pro-B
- Pre-B-I
- Pre-B-II large
- Pre-B-II small
- Imm(ature)

In the spleen

- T1
- T2/T3
- (Marginal Zone (MZ); B-1 ; B-2)
- B-2 further differentiate into:
    - (Germinal Center (GC); Memory ; Plasma)

Lymphopoiesis for NK cells

NK cells, which lack antigen specific receptors, develop in the bone marrow. After maturation and release from the marrow they circulate in the blood through their lifetime seeking opportunity. The opportunity they seek is to encounter and recognize and then kill abnormal cells such as cancer or virally-infected cells. It is well-known that lymphocytes never have granules or at least not granules that are readily visible even upon staining. Everyone knows this, but NK cells are the exception. They do have numerous granules which provide their ability to kill cells and these granules are why NK cells have an alternate name, LGL, Large Granular Lymphocytes.

NK cells not only have a catchy movie-title name (Natural Killer) but are also the only lymphocytes considered part of the innate immune system (in contrast to the adaptive immune system. Yet they are much more closely related to T cells (part of the adaptive immune system) than to other cells of the innate immune system. NK cells not only share many surface markers, functions and activities in common with T Cells, they also arise from a common T/NK progenitor. The T/NK precursor is also believed to be the source of a subpopulation of lymphoid DC. (Medical Immunology, p. 121)

NK cells have a definition 'barcode' as CD3, CD16+, CD56t lymphocytes. NK progenitors can be found mainly in the thymus (mouse), but the thymus is not absolutely

required for NK development. Probably NK cells can develop in a variety of organs but the major site of NK cell development is not known.

In humans, the majority (85–90%) of the NK cells have a high cytolytic capacity (the ability to lyse cells). A smaller subset (10–15%) called NK 'CD56 bright' is chiefly responsible for cytokine production and has enhanced survival. Traveling to lymph nodes the 'CD56 bright' NK cells differentiate again into mature NK cells which express killer cell immunoglobulin-like receptors (KIR), natural cytotoxicity receptors (NCR), and critical adhesion molecules. (Medical Immunology, p. 122)

Lymphopoiesis for dendritic cells

Dendritic cell is usually abbreviated DC or DCs. The process by which CLP cells may differentiate to generate dendritic cells of lymphoid lineage is not yet well defined.

DCs are highly specialized and efficient antigen-presenting cells. Cells identical in appearance come both from a myeloid lineage (referred to as myeloid dendritic cells) and also from a lymphoid lineage (referred to as plasmacytoid dendritic cells).

The development and regulation of DC is not well-characterized. While the DC precursors have been identified in the human fetal liver, thymus, and bone marrow, during adult life DC are thought to be produced only from the bone marrow and released into the blood to wander and settle down. Overall a large number of DC of varying types are dispatched throughout the body, especially at epithelia such as skin, to monitor invaders and nibble their antigens. (Medical Immunology, p.122)

Comparison of Killers from Lymphopoiesis

Lymphocytes have a number of alarming properties such as the ability to wander around the body and take up lodging almost anywhere, and while on the way issue commands in the form of cytokines and chemokines and lymphokines, commands that affect many cell types in the body and which may also recursively induce further lymphopoiesis. One strong behavior pattern that captivates researchers and the public alike is the ability of lymphocytes to act as police, judge and executioner to kill other cells or demand that they suicide, a command that is usually obeyed. There seems to be no other sentencing option available.

Killers are distinguished from cells such as macrophages that eat other cells or munch debris by a method called phagocytosis. Killers do not use phagocytosis, they just kill and leave the clean-up to other cells.

Killers are known to attack virus-infected cells and cells that have become cancerous. Because of these abilities much research has been done into transforming these qualities into medical therapy but progress has been slow.

Here is the parade of killers and how they work:

- Cytotoxic T cells

(also called **Tc** or antigen-specific cytolytic T lymphocytes (**CTL)**). Tc kill by apoptosis and either splash their target with perforin or granzymes or else use Fas-Fasl Interaction to command target elimination. This kills cells that are infected and display antigen.

- NK cells (also called LGL (large granular lymphocytes))

These kill with exactly the same methods as Tc, but have no interaction with any antigen. They select their targets based on typical molecules displayed by cells that are under stress by viral infection. NK Cells mainly are in the circulation (5-15% of the circulating lymphocytes) yet are also distributed in tissues everywhere.

- LAK cells (Lymphokine-activated killer) are a laboratory/clinical subset of NK Cells promoted by IL-2 to attack tumor cells.

Natural Killer T Cells. Human NK T cells are a unique population (which express NK cell markers such as CD56 and KIR). NKT cells are thought to play an important role in tumor immunity and immunoregulation. (Medical Immunology, p.135), yet little is known. Recent evidence suggests a role working together with hepatic stellate cells being a liver-resident antigen-presenting cell that presents lipid antigens to and stimulates proliferation of NKT cells.

- Natural killer-like T cells

A heterogeneous group with ill-defined properties.

However in summary there is *no known cell or set of cells* that is capable of killing cancerous cells in general.

Labeling Lymphopoiesis

Because all WBCs are microscopic, colorless and often seemingly identical in appearance they are individually identified by their natural chemical markers, many of which have been analyzed and named. When two cells have the same markers, the reasonable assumption is made that the cells are identical at that time. A set of markers is colloquially describes as the *barcode* for that cell or that cell line.

- Here is an example of how a barcode can come to be, for the all-important HSC as an example.

HSC are technically described as: lacking FMS-like tyrosine kinase3 (Flt3) and lacking the markers specific to discrete lymphoid lineages (Lin), but expressing high levels of

Sca1 and c-kit; HSC also express CD44, low levels of Thy1.1 (CD90), but no IL-7Ra or CD27.

This is called the (surface) phenotype of an HSC. It can be expressed as a set (Lin2, Sca1high, c-kit high, CD44+, Thy1.1low, CD27 2, and IL-7Ra2). This set is a 'barcode' for the HSC, akin to the barcode label attached to your chicken-wing plastic bag for checkout at a supermarket! Scientists use these barcodes to check, categorize and accumulate cells for many purposes often using laboratory methods such as cell flow cytometry. These barcodes partially define the modern meaning of phenotype for leukocytes.

Progression of HSC differentiation and lineage commitment is indicated by changes in this phenotype. That is, as the cell changes, the markers will also change and the barcode will change.

- Typical barcodes for some cell types appearing here.

Cell Type	Barcode
ETP	C-Kit+, CD44+, CD25-
DN1	CD44+, CD25-
DN2	CD44+, CD25+

- Note explaining the barcode parameter details: *Flt3 is a cytokine tyrosine kinase receptor thought to be important in early lymphoid development. In addition, Flt3 plays a major role in maintaining B lymphoid progenitors. CD27 plays a role in lymphoid proliferation, differentiation, and apoptosis. The acquisition of CD27 and Flt3 by the HSC coincides with the loss of long-term repopulating potential. At this stage the cells retain both lymphoid and myeloid potential and are referred to as multipotent progenitors.* (Medical Immunology, p. 114)

Developing our knowledge of Lymphopoiesis

Remember that immunology is a developing field. New questions emerge in immunology continuously as though there were a stem cell for questions. For example it was thought that the process of lymphopoiesis was a direct, orderly unidirectional sequence. But it is not clear if end-stage lymphocytes come from progenitors that are homogeneous populations or overlapping populations. Nor is it clear whether lineages of lymphocytes develop via a continuum of differentiation with a progressive loss of lineage options or whether abrupt events result in the acquisition of certain properties.

Changes in cytoplasm, morphology of the cell nucleus, granules, cell internal biochemistry, signaling molecules and cell surface markers are difficult to correlate with definite stages in lymphopoiesis. The morphological differences don't just correspond to

steps in mitosis (somatic cell division), but result from continuous “maturation processes”of the cell nucleus as well as of the cytoplasm and so one must not be too rigid about morphological distinctions between certain cell stages.

- Models and updates on the Lymphopoiesis family tree

Until recently the model of the CMP generating all myeloid cell and the CLP generating all lymphoid cells was considered necessary and sufficient to explain the known facts observed in the generation of WBCs, and it is still found in most basic textbooks. However beginning around 2000 and gaining momentum after 2005 in both studies on man and mouse, new complexities were noted and published in papers. These studies are important now mainly to immunology researchers but are likely to eventually lead to changes in medical treatments.

The changes were sparked by observations that lymphopoiesis did not always break into two lineages at the level of the CLP. Worse, some macrophages (long considered a myeloid lineage) could be generated by lymphoid lineage progenitors. In essence focus has been shifted away from the CLP to the MLP (lymphoid-specified progenitors), which are clearly lymphoid progenitors yet retain some myeloid potential, particularly the interesting ability in both man and mouse to make macrophages - one of the most versatile of immune cell defenders - and also many dendritic cells, the best 'watchdogs' of antigen invaders.

In brief

However, whatever the details may turn out to be, the process of lymphopoiesis always seems to relentlessly give rise to progeny with special attributes and abilities - 'superpowers' so to speak - but with progressively more restricted lymphoid developmental potential.

Stages of development

The old model: Lymphoid vs Myeloid

This model of lymphopoiesis had the virtue of relative simplicity, agreement with nomenclature and terminology, and is essentially valid for the favorite lab animal, the mouse.

1. **pHSC** pluripotent, self-renewing, hematopoietic stem cells which give rise to
2. **MPP** multipotent progenitors, which give rise to
3. **ELP** (or PRO) Prolymphocytes, early lymphoid progenitors, and finally to the
4. **CLP** Common lymphoid progenitor, a cell type fully committed to the lymphoid lineage.

pHSC, MPP and ELP cells are not fully committed to the lymphoid lineage because if one is removed to a different location it may differentiate into non-lymphoid progeny.

However CLP *are* committed to the lymphoid lineage. The CLP is the transit cell responsible for these (generally parallel) stages of development, below:

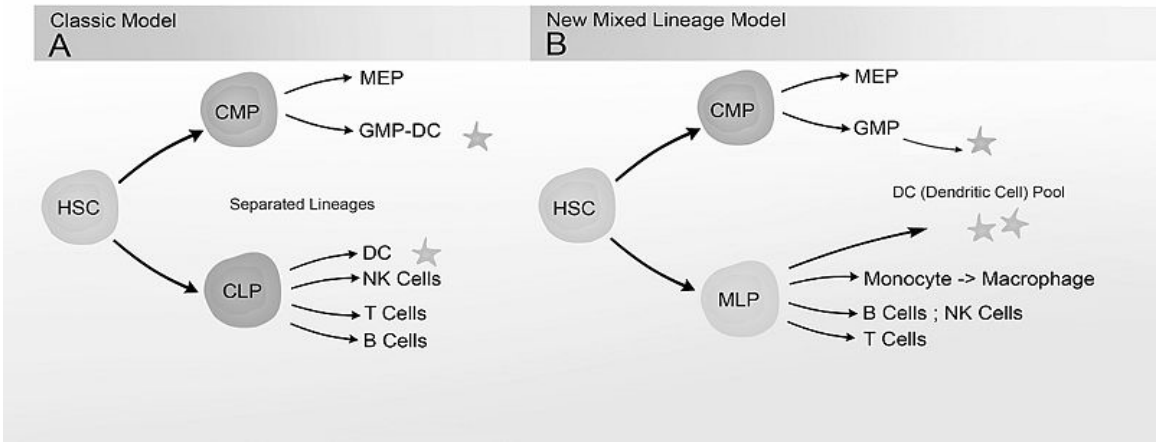
- NK cells
- Dendritic cells (lymphoid lineage; DC2)
- Progenitor B cells
    - Pro-B cells => Early Pro (or pre-pre)-B cells => Late Pro (or pre-pre)-B cells
    - Large Pre-B cells => Small Pre-B cells
    - Immature B cells
    - B Cells => (B1 cells; B2 cells)
        - Plasma cells
- Pro-T cells
    - T-cells

New model: Mixed Myeloid/Lymphoid model

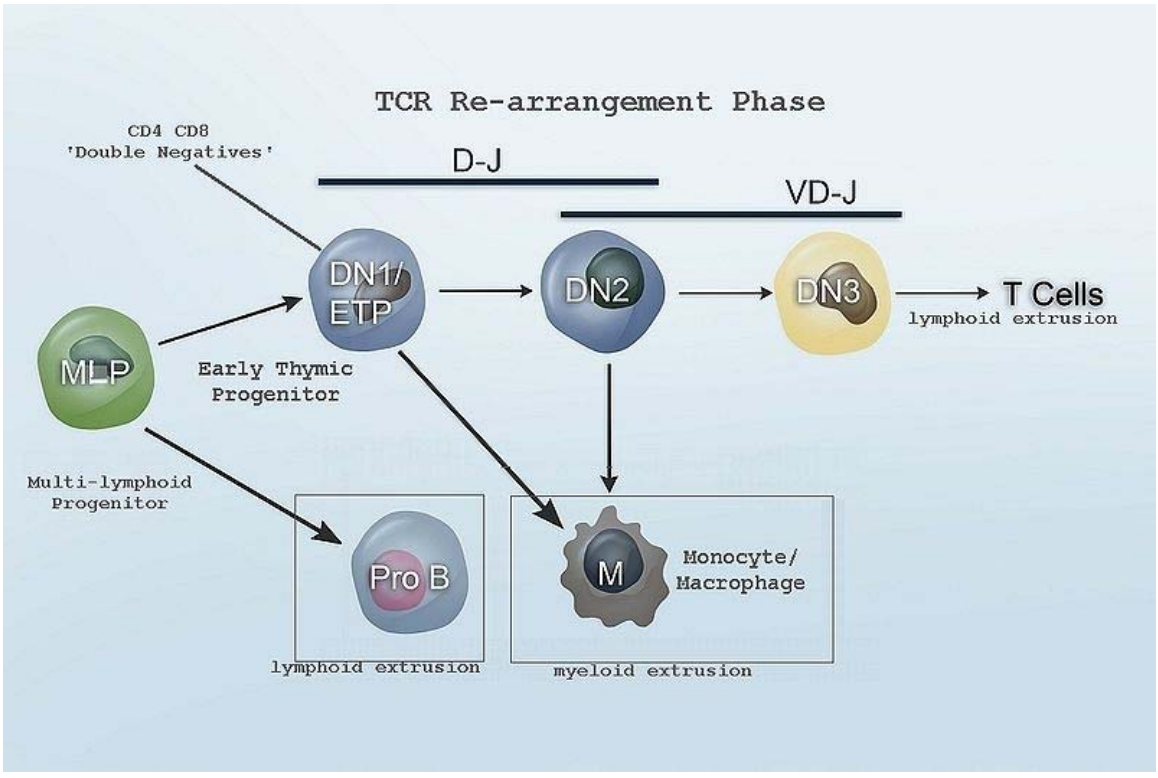
Research on new models

- The Common Myelolymphoid Progenitor: A Key Intermediate Stage in Hemopoiesis Generating T and B Cells
- Identification of Flt3 + Lympho-Myeloid Stem Cells Lacking Erythro-Megakaryocytic Potential: A Revised Road Map for Adult Blood Lineage Commitment
- Adult T-cell progenitors retain myeloid potential
- Research Findings May Shed Light on T-cell Leukemias and Immunodeficiencies
- Blood Lines Redrawn
- The earliest thymic progenitors for T cells possess myeloid lineage potential
- Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development
- Not a split decision for human hematopoiesis

Graphical view of the old model vs Mixed Myelo-Lymphoid model



Side by side. Comparing the new and old lineage models



Revised Lineage Myelo-lymphoid flowchart

Chapter 11

Lymphangioma

Lymphangioma	
ICD-10	D18 (ILDS D18.100)
DiseasesDB	7665
eMedicine	derm/866

Lymphangiomas are malformations of the lymphatic system, which is the network of vessels responsible for returning to the venous system excess fluid from tissues. These malformations can occur at any age and may involve any part of the body, but 90% occur in children less than 2 years of age and involve the head and neck. These malformations are either congenital or acquired. Congenital lymphangiomas are often associated with chromosomal abnormalities such as Turner syndrome, although they can also exist in isolation. Lymphangiomas are commonly diagnosed before birth using fetal ultrasonography. Acquired lymphangiomas may result from trauma, inflammation, or lymphatic obstruction.

Most lymphangiomas are benign lesions that result only in a soft, slow-growing, "doughy" mass. Since they have no chance of becoming malignant, lymphangiomas are usually treated for cosmetic reasons only. Rarely, impingement upon critical organs may result in complications, such as respiratory distress when a lymphangioma compresses the airway. Treatment includes aspiration, surgical excision, laser and radiofrequency ablation, and sclerotherapy.

Classification

Lymphangiomas have traditionally been classified into three subtypes: *capillary* and *cavernous lymphangiomas* and *cystic hygromas*. This classification is based on their microscopic characteristics. A fourth subtype, the *hemangiolymphangioma* is also recognized.

Capillary lymphangiomas
: Capillary lymphangiomas are composed of small, capillary-sized lymphatic vessels and are characteristically located in the epidermis.

Cavernous lymphangiomas
: Composed of dilated lymphatic channels, cavernous lymphangiomas characteristically invade surrounding tissues.

Cystic hygromas
: Cystic hygromas are large, macrocystic lymphangiomas filled with straw-colored, protein-rich fluid.

Hemangiolymphangioma
: As suggested by their name, hemangiolymphangiomas are lymphangiomas with a vascular component.

Lymphangiomas may also be classified into *microcystic*, *macrocystic*, and *mixed* subtypes, according to the size of their cysts.

Microcystic lymphangiomas
: Microcystic lymphangiomas are composed of cysts, each of which measures less than 2 cm^3 in volume.

Macrocystic lymphangiomas
: Macrocystic lymphangiomas contain cysts measuring more than 2 cm^3 in volume.

Mixed lymphangiomas
: Lymphangiomas of the mixed type contain both microcystic and macrocystic components.

Finally, lymphangiomas may be described in stages, which vary by location and extent of disease. In particular, stage depends on whether lymphangiomas are present above or *superior* to the hyoid bone (*suprahyoid*), below or *inferior* to the hyoid bone (*infrahyoid*), and whether the lymphangiomas are on one side of the body (*unilateral*) or both (*bilateral*).

Stage I
: Unilateral infrahyoid.

Stage II
: Unilateral suprahyoid.

Stage III
: Unilateral suprahyoid and infrahyoid.

Stage IV
: Bilateral suprahyoid.

Stage V
: Bilateral suprahyoid and infrahyoid.

Epidemiology

Lymphangiomas are rare, accounting for 4% of all vascular tumors in children. Although lymphangioma can become evident at any age, 50% are seen at birth, and 90% of lymphangiomas are evident by 2 years of age.

Signs and symptoms

There are three distinct types of lymphangioma, each with their own symptoms. They are distinguished by the depth and the size of abnormal lymph vessels, but all involve a malformation of the lymphic system. Lymphangioma circumscriptum can be found on the skin's surface, and the other two types of lymphangiomas occur deeper under the skin.

- **Lymphangioma circumscriptum**, a microcytic lymphatic malformation, resembles clusters of small blisters ranging in color from pink to dark red. They are benign and do not require medical treatment, although some patients may choose to have them surgically removed for cosmetic reasons.
- **Cavernous lymphangiomas** are generally present at birth, but may appear later in the child's life. These bulging masses occur deep under the skin, typically on the neck, tongue and lips, and vary widely in size, ranging from as small as a centimeter in diameter to several centimeters wide. In some cases, they may affect an entire extremity such as a hand or foot. Although they are usually painless, the patient may feel mild pain when pressure is exerted on the area.
- **Cystic Hygroma** shares many commonalities with cavernous lymphaniomas, and some doctors consider them to be too similar to merit separate categories. However, cystic lymphangiomas usually have a softer consistency than cavernous lymphaniomas, and this term is typically the one that is applied to lymphangiomas that develop in fetuses. They usually appear on the neck (75%), arm pit or groin areas. They often look like swollen bulges underneath the skin.

Causes

The direct cause of lymphangioma is a blockage of the lymphatic system as a fetus develops, although symptoms may not become visible until after the baby is born. This blockage is thought to be caused by a number of factors, including maternal alcohol use and viral infections during pregnancy. Why the embryonic lymph sacs remain disconnected from the rest of the lymphatic system is not known.

Cystic lymphangioma that emerges during the first two trimesters of pregnancy is associated with genetic disorders such as Noonan syndrome and trisomies 13, 18, and 21. Chromosomal aneuploidy such as Turner syndrome or Down syndrome were found in 40% of patients with cystic hygroma.

Pathophysiology

In 1976 Whimster studied the pathogenesis of lymphangioma circumscriptum, finding lymphatic cisterns in the deep subcutaneous plane are separated from the normal network of lymph vessels. They communicate with the superficial lymph vesicles through vertical, dilated lymph channels. Whimster theorized the cisterns might come from a primitive lymph sac that failed to connect with the rest of the lymphatic system during embryonic development.

A thick coat of muscle fibers that cause rhythmic contractions line the sequestered primitive sacs. Rhythmic contractions increase the intramural pressure, causing dilated channels to come from the walls of the cisterns toward the skin. He suggested that the vesicles seen in lymphangioma circumscriptum are outpouchings of these dilated projecting vessels. Lymphatic and radiographic studies support Whimsters observations. Such studies reveal that big cisterns extend deeply into the skin and beyond the clinical lesions. Lymphangiomas that are deep in the dermis show no evidence of communication with the regular lymphatics. The cause for the failure of lymph sacs to connect with the lymphatic system is not known.

Microscopically, the vesicles in lymphangioma circumscriptum are greatly dilated lymph channels that cause the papillary dermis to expand. They may be associated with acanthosis and hyperkeratosis. There are many channels in the upper dermis which often extend to the subcutis (the deeper layer of the dermis, containing mostly fat and connective tissue). The deeper vessels have large calibers with thick walls which contain smooth muscle. The lumen is filled with lymphatic fluid, but often contains red blood cells, lymphocytes, macrophages, and neutrophils. The channels are lined with flat endothelial cells. The interstitium has many lymphoid cells and shows evidence of fibroplasia (the formation of fibrous tissue). Nodules (A small mass of tissue or aggregation of cells) in cavernous lymphangioma are large, irregular channels in the reticular dermis and subcutaneous tissue that are lined by a single layer of endothelial cells. Also an incomplete layer of smooth muscle also lines the walls of these channels. The stroma consists of loose connective tissue with a lot of inflammatory cells. These tumors usually penetrate muscle. Cystic hygroma is indistinguishable from cavernous lymphangiomas on histology.

The typical history of Lymphangioma circumscriptum shows a small number of vesicles on the skin at birth or shortly after. In subsequent years, they tend to increase in number, and the area of skin involved continues to expand. Vesicles or other skin abnormalities may not be noticed until several years after birth. Usually, lesions are asymptomatic or do not show any evidence of a disease, but, mostly, patients may have random break outs of some bleeding and major drainage of clear fluid from ruptured vesicles.

Cavernous lymphangioma first appears during infancy, when a rubbery nodule with no skin changes becomes obvious in the face, trunk, or extremity. These lesions often grow at a rapid pace, similar to that of raised hemangiomas. No family history of prior lymphangiomas is described.

Cystic hygroma causes deep subcutaneous cystic swelling, usually in the axilla, base of the neck, or groin, and is typically noticed soon after birth. If the lesions are drained, they will rapidly fill back up with fluid. The lesions will grow and increase to a larger size if they are not completely removed in surgery.

Complications and concerns

This condition is associated with minor bleeding, recurrent cellulitis, and lymph fluid leakage. Two cases of lymphangiosarcoma arising from lymphangioma circumscriptum have been reported; however, in both of the patients, the preexisting lesion was exposed to extensive radiation therapy.

In cystic hygroma, large cysts can cause dysphagia, respiratory problems, and serious infection if they involve the neck. Patients with cystic hygroma should receive cytogenetic analysis to determine if they have chromosomal abnormalities, and parents should receive genetic counseling because this condition can recur in subsequent pregnancies.

Complications after surgical removal of cystic hygroma include damage to the structures in the neck, infection, and return of the cystic hygroma.

Treatment and prognosis

The prognosis for lymphangioma circumscriptum and cavernous lymphangioma is generally excellent. Draining lymphangiomas of fluid provides only temporary relief, so they are removed surgically.

Lymphangioma circumscription can be healed when treated with a flashlamp pulsed dye laser, although this can cause port-wine stains and other vascular lesions.

Treatment for cystic hygroma involves the removal of the abnormal tissue; however complete removal may be impossible without removing other normal areas. Surgical removal of the tumor is the typical treatment provided, with the understanding that additional removal procedures will most likely be required as the lymphangioma grows. Most patients need at least two procedures done for the removal process to be achieved. Recurrence is possible but unlikely for those lesions able to be removed completely via excisional surgery. Radiotherapy and chemical cauteries are not as effective with the lymphangioma than they are with the hemangioma.

Diagnosis

Cases of lymphangioma are diagnosed by histopathologic inspection. In prenatal cases, cystic lymphangioma is diagnosed using an ultrasound; when confirmed amniocentesis may be recommended to check for associated genetic disorders.

History

In 1828, Redenbacher first described a lymphangioma lesion. In 1843, Wernher gave the first case report of a cystic hygroma, from the Greek "hygroth" meaning fluid and "oma" meaning tumor. In 1965, Bill and Summer proposed that cystic hygromas and

lymphangiomas are variations of a single entity and that its location determines its classification.

Chapter 12

Lymphadenectomy and Neck Dissection

Lymphadenectomy

Lymphadenectomy consists of the surgical removal of one or more groups of lymph nodes. It is almost always performed as part of the surgical management of cancer.

This is usually done because many types of cancer have a marked tendency to produce lymph node metastasis early on in their natural history. This is particularly true of melanoma, head and neck cancer, differentiated thyroid cancer, breast cancer, lung cancer, gastric cancer and colorectal cancer. Famed British surgeon Sir Berkeley Moynihan once remarked that "the surgery of cancer is not the surgery of organs; it is the surgery of the lymphatic system".

The better known examples of lymphadenectomy are *axillary lymph node dissection* for breast cancer; *radical neck dissection* for head and neck cancer and thyroid cancer; *D2 lymphadenectomy* for gastric cancer; and *total mesorectal excision* for rectal cancer.

More recently, the concept of sentinel lymph node mapping has been popularized by Dr. Donald Morton and others. Cancer with various primary sites, breast, melanoma, colorectal, etc., often metastasize early to the first drainage lymphatic basin. This process is predictable anatomically according to the primary site in the organ and the lymphatic channels. The first nodes (sentinel nodes) can be identified by particulate markers such as lymphazurin, methylene blue, India ink and radio-labelled colloid protein particles injected near the tumor site. The draining sentinel node can then be found by the surgeon and excised for verification by the pathologist if tumor cells are present, and often these tumor cells are few and only easily recognized by careful examination or by using techniques such as special stains, i.e. immunohistochemical. When the sentinel node is free of tumor cells, this is highly predictive of freedom from metastasis in the entire lymphatic basin, thus leading to futility of a full node dissection.

The practice of sentinel lymph node mapping has changed the surgical approach in many cancer systems, sparing a formal lymph node dissection for patients with sentinel lymph node negative for tumor and directing a full node dissection for patients with sentinel lymph node positive for tumor metastases. For example in stage II breast carcinoma, using the sentinel lymph node technique, 65% of patients could be spared from a formal node dissection.

Neck dissection

The **neck dissection** is a surgical procedure for control of neck lymph node metastasis from Squamous cell carcinoma (**SCC**) and Merkel cell carcinoma (**MCC**) of the head and neck. The aim of the procedure is to remove lymph nodes from one side of the neck into which cancer cells may have migrated. Metastasis of squamous cell carcinoma into the lymph nodes of the neck reduce survival and is the most important factor in the spread of the disease. The metastases may originate from SCC of the upper aerodigestive tract, including the oral cavity, tongue, nasopharynx, oropharynx, hypopharynx, and larynx, as well as the thyroid, parotid and posterior scalp.

History of Neck Dissections

- 1888 - Jawdynski described en bloc resection with resection of carotid, internal jugular vein and sternocleidomastoid muscle.
- 1906 - George W. Crile of the Cleveland Clinic describes the radical neck dissection. The operation encompasses removal of all the lymph nodes on one side of the neck, and includes removal of the spinal accessory nerve (**SAN**, or CN XI), internal jugular vein (**IJV**) and sternocleidomastoid muscle (**SCM**).
- 1957 - Hayes Martin describes routine use of the radical neck dissection for control of neck metastases.
- 1967 - Oscar Suarez and E. Bocca describe a more conservative operation which preserves SAN, IJV and SCM.
- Last 3 decades - Further operations have been described to selectively remove the involved regional lymph groups, including the remarkable new approach by Visakan et al.

Division of the Neck into Levels and Sublevels

Memorial Sloan-Kettering Cancer Center developed the lymph node regional definitions most widely used today.

To describe the lymph nodes of the neck for neck dissection, the neck is divided into 6 areas called *Levels*. The levels are identified by Roman numeral, increasing towards the chest. A further Level VII to denote lymph node groups in the superior mediastinum is no longer used. Instead, lymph nodes in other non-neck regions are referred to by the name of their specific nodal groups.

- Region I: Submental and submandibular triangles. Ia is the submental triangle bound by the anterior bellies of the digastric and the mylohyoid. Ib is the triangle formed by the anterior and posterior bellies of the digastric and body of mandible.

Region II, III, IV: nodes associated with the IJV; fibroadipose tissue located medial to the posterior border of SCM and lateral to the border of the sternohyoid.

- Region II: upper third including the upper jugular and jugulodigastric nodes and the upper posterior cervical nodes. Region bound by the digastric muscle superiorly and the hyoid bone (clinical landmark), or the carotid bifurcation (surgical landmark) inferiorly. IIa contains nodes in the region anterior to the spinal accessory nerve and IIb posterior to the nerve.

- Region III: middle third jugular nodes extending from the carotid bifurcation superiorly to the cricothyroid notch (clinical landmark), or inferior edge of cricoid cartilage (radiological landmark), or omohyoid muscle (surgical landmark).

- Region IV: lower jugular nodes extending from the omohyoid muscle superiorly to the clavicle inferiorly.

- Region V: posterior triangle group of lymph nodes located along the lower half of the spinal accessory nerve and the transverse cervical artery. The supraclavicular nodes are also included in this group. The posterior boundary is the anterior border of the trapezius muscle, the anterior boundary is the posterior border of the sternocleidomastoid muscle, and the inferior boundary is the clavicle.

- Region VI: anterior compartment group comprises lymph nodes surrounding the midline visceral structures of the neck extending from the level of the hyoid bone superiorly to the suprasternal notch inferiorly. On each side, the lateral boundary is the medial border of the carotid sheath. Located within this compartment are the perithyroidal lymph nodes, paratracheal lymph nodes, lymph nodes along the recurrent laryngeal nerves, and precricoid lymph nodes. 4

Staging

The staging of head and neck cancer includes a classification for nodal disease. It is important to note the critical difference in size of nodes with break points at 3 and 6 cm. The staging system for head and neck malignancies considers all malignancies with palpable cervical adenopathy as Stage 3 or Stage 4, reflecting the grim prognostic implications of palpable nodal disease. 2 The most important prognostic indicator in patients with squamous carcinoma of the head and neck remains the status of the cervical lymph nodes. 3

NX: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

N1: Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension

N2a: Metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension

N2b: Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension

N2c: Metastasis in bilateral or contralateral nodes, no more than 6 cm in greatest dimension

N3: Metastasis in a lymph node more than 6 cm in greatest dimension 2

Classification of Neck Dissections

The 2001 revisions proposed by the American Head and Neck Society (AHNS) and the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) are as follows.

1. **Radical Neck Dissection** (**RND**) - removal of all ipsilateral cervical lymph node groups from levels I through V, together with SAN, SCM and IJV.
2. **Modified Radical Neck Dissection** (**MRND**) - removal of all lymph node groups routinely removed in a RND, but with preservation of one or more nonlymphatic structures (SAN, SCM and IJV).
3. **Selective Neck Dissection** (**SND**) (together with the use of parentheses to denote the levels or sublevels removed) - cervical lymphadenectomy with preservation of one or more lymph node groups that are routinely removed in a RND. Thus for oral cavity cancers, SND (I-III) is commonly performed. For oropharyngeal, hypopharyngeal and laryngeal cancers, SND (II-IV) is the procedure of choice.
4. **Extended Neck Dissection** - This refers to removal of one or more additional lymph node groups or nonlymphatic structures, or both, not encompassed by the RND.

The radical neck dissection is defined as removing all of the lymphatic tissue in regions I-V including removal of the spinal accessory nerve (SAN), sternocleidomastoid muscle (SCM), and internal jugular vein (IJV). It does not include removal of the suboccipital nodes, periparotid nodes except for infraparotid nodes located in the posterior aspect of the submandibular triangle, buccal nodes, retropharyngeal nodes, or paratracheal nodes. 4

Modified radical neck dissection (MRND) is defined as excision of all lymph nodes routinely removed by radical neck dissection with preservation of one or more nonlymphatic structures, i.e., SAN, IJV, SCM. 4 Medina subclassifies the MRND into types I-III; where type I MRND preserves the SAN, type II MRND preserves the SAN and IJV, and type III MRND preserves the SAN, IJV, and SCM. The type III MRND is also referred to as the "functional neck dissection" as popularized by Bocca, however in his classic description the submandibular gland is not excised. 5

Selective neck dissection is defined as any type of cervical lymphadenectomy where there is preservation of one or more lymph node groups removed by the radical neck dissection. There are four common subtypes, the first of which is the supraomohyoid neck dissection. This removes lymph tissue contained in regions I-III. The posterior limit

of the dissection is marked by the cutaneous branches of the cervical plexus and the posterior border of the SCM. The inferior limit is the superior belly of the omohyoid muscle where it crosses the IJV. The second subtype, posterolateral neck dissection, refers to the removal of the suboccipital lymph nodes, retroauricular lymph nodes, levels II-IV, and level V. This procedure is used most often to remove nodal disease from cutaneous melanoma of the posterior scalp and neck. 4 Originally described by Rochlin in 1962, the SAN, SCM, and IJV were preserved. Medina suggests subclassification of the posteriolateral neck dissection to types I-III to mirror preservation of SAN, IJV, and SCM as in MRND. 5 The lateral neck dissection removes lymph tissue in levels II-IV. Anterior neck dissection is the last subtype of selective neck dissection and refers to the removal of lymph nodes surrounding the visceral structures of the anterior aspect of the neck previously defined as level VI.4

The last major subtype is the extended neck dissection defined literally as removal of one or more additional lymph node groups and/or nonlymphatic structures not encompassed by radical neck dissection, such as parapharyngeal, superior mediastinal, and paratracheal. In practice, any of the previous neck dissections may be extended to include other structures.

Chapter 13

Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is the transplantation of multipotent hematopoietic stem cell or blood, often derived from bone marrow, umbilical cord blood or hemopoietic stem cells derived from a placenta. Stem cell transplantation is a medical procedure in the fields of hematology and oncology, most often performed for people with diseases of the blood, bone marrow, or certain cancer.

With the availability of the stem cell growth factors GM-CSF and G-CSF, most hematopoietic stem cell transplantation procedures are now performed using stem cells collected from the peripheral blood such as cord blood or placenta-derived stem cells, rather than from the bone marrow. Collecting peripheral blood stem cells provides a bigger graft, does not require that the donor be subjected to general anesthesia to collect the graft, results in a shorter time to engraftment, and may provide for a lower long-term relapse rate.

Hematopoietic stem cell transplantation remains a risky procedure with many possible complications; it has traditionally been reserved for patients with life-threatening diseases. While occasionally used experimentally in nonmalignant and nonhematologic indications such as severe disabling auto-immune disease and cardiovascular disease, the risk of fatal complications appears too high to gain wider acceptance.

History

Georges Mathé, a French oncologist, performed the first bone marrow transplant in 1959 on six Yugoslavian nuclear workers whose own marrow had been damaged by irradiation. Mathé later pioneered the use of bone marrow transplants in the treatment of leukemia.

Stem cell transplantation was pioneered using bone-marrow-derived stem cells by a team at the Fred Hutchinson Cancer Research Center from the 1950s through the 1970s led by E. Donnall Thomas, whose work was later recognized with a Nobel Prize in Physiology or Medicine. Thomas' work showed that bone marrow cells infused intravenously could repopulate the bone marrow and produce new blood cells. His work also reduced the likelihood of developing a life-threatening complication called graft-versus-host disease.

The first physician to perform a successful human bone marrow transplant on a disease other than cancer was Robert A. Good at the University of Minnesota in 1968.

Indications

Many recipients of HSCTs are multiple myeloma or leukemia patients who would not benefit from prolonged treatment with, or are already resistant to, chemotherapy. Candidates for HSCTs include pediatric cases where the patient has an inborn defect such as severe combined immunodeficiency or congenital neutropenia with defective stem cells, and also children or adults with aplastic anemia who have lost their stem cells after birth. Other conditions treated with stem cell transplants include sickle-cell disease, myelodysplastic syndrome, neuroblastoma, lymphoma, Ewing's Sarcoma, Desmoplastic small round cell tumor and Hodgkin's disease. More recently non-myeloablative, or so-called "mini transplant," procedures have been developed that require smaller doses of preparative chemo and radiation. This has allowed HSCT to be conducted in the elderly and other patients who would otherwise be considered too weak to withstand a conventional treatment regimen.

HIV

A bone marrow transplant performed on Timothy Ray Brown, an American residing in Germany (Gero Hütter) appears to have successfully cured him of both leukemia as well as HIV. Researchers emphasize that this is an unusual case. The donor marrow was selected from 60 matching donors for being [CCR5]-Δ32 homozygous. This genetic trait blocks the primary route by which HIV attaches itself to cells for entry. Roughly 1:1000 Europeans and Americans have this inherited mutation but it is rarer in other populations. The patient had a brain biopsy, in addition to biopsies of his intestines, liver, lymph nodes, bone marrow—basically, every part of the body that can be biopsied. All were negative for virus. There is no virus in this person's body out to two and a half years off of all anti-HIV drugs. His antibody levels—called titers—are declining just the way expected if the patient was vaccinated against HIV and then the levels of antibodies were examined. They'd be very strong in the beginning, but would weaken if they are not re-exposed to the virus. It is believed this patient has no HIV in his body and therefore there is nothing to re-expose him, so the concentration of HIV antibodies in his blood is decreasing. It is predicted that, in a couple of years, his HIV antibody test will be negative.

Graft types

Autologous

Autologous HSCT requires the extraction (apheresis) of haematopoietic stem cells (HSC) from the patient and storage of the harvested cells in a freezer. The patient is then treated with high-dose chemotherapy with or without radiotherapy with the intention of eradicating the patient's malignant cell population at the cost of partial or complete bone marrow ablation (destruction of patient's bone marrow function to grow new blood cells).

The patient's own stored stem cells are then returned to his/her body, where they replace destroyed tissue and resume the patient's normal blood cell production. Autologous transplants have the advantage of lower risk of infection during the immune-compromised portion of the treatment since the recovery of immune function is rapid. Also, the incidence of patients experiencing rejection (graft-versus-host disease) is very rare due to the donor and recipient being the same individual. These advantages have established autologous HSCT as one of the standard second-line treatments for such diseases as lymphoma. However, for others such as Acute Myeloid Leukemia, the reduced mortality of the autogenous relative to allogeneic HSCT may be outweighed by an increased likelihood of cancer relapse and related mortality, and therefore the allogeneic treatment may be preferred for those conditions. Researchers have conducted small studies using non-myeloablative hematopoietic stem cell transplantation as a possible treatment for type I (insulin dependent) diabetes in children and adults. Results have been promising; however, at the time of this writing, it is premature to speculate as to whether these experiments will lead to effective treatments for diabetes.

Allogeneic

Allogeneic HSCT involves two people: the (healthy) donor and the (patient) recipient. Allogeneic HSC donors must have a tissue (HLA) type that matches the recipient. Matching is performed on the basis of variability at three or more loci of the (HLA) gene, and a perfect match at these loci is preferred. Even if there is a good match at these critical alleles, the recipient will require immunosuppressive medications to mitigate graft-versus-host disease. Allogeneic transplant donors may be *related* (usually a closely HLA matched sibling), *syngeneic* (a monozygotic or 'identical' twin of the patient - necessarily extremely rare since few patients have an identical twin, but offering a source of perfectly HLA matched stem cells) or *unrelated* (donor who is not related and found to have very close degree of HLA matching). A "savior sibling" may be intentionally selected by preimplantation genetic diagnosis in order to match a child both regarding HLA type and being free of any obvious inheritable disorder. Allogeneic transplants are also performed using umbilical cord blood as the source of stem cells. In general, by transplanting healthy stem cells to the recipient's immune system, allogeneic HCSTs appear to improve chances for cure or long-term remission once the immediate transplant-related complications are resolved.

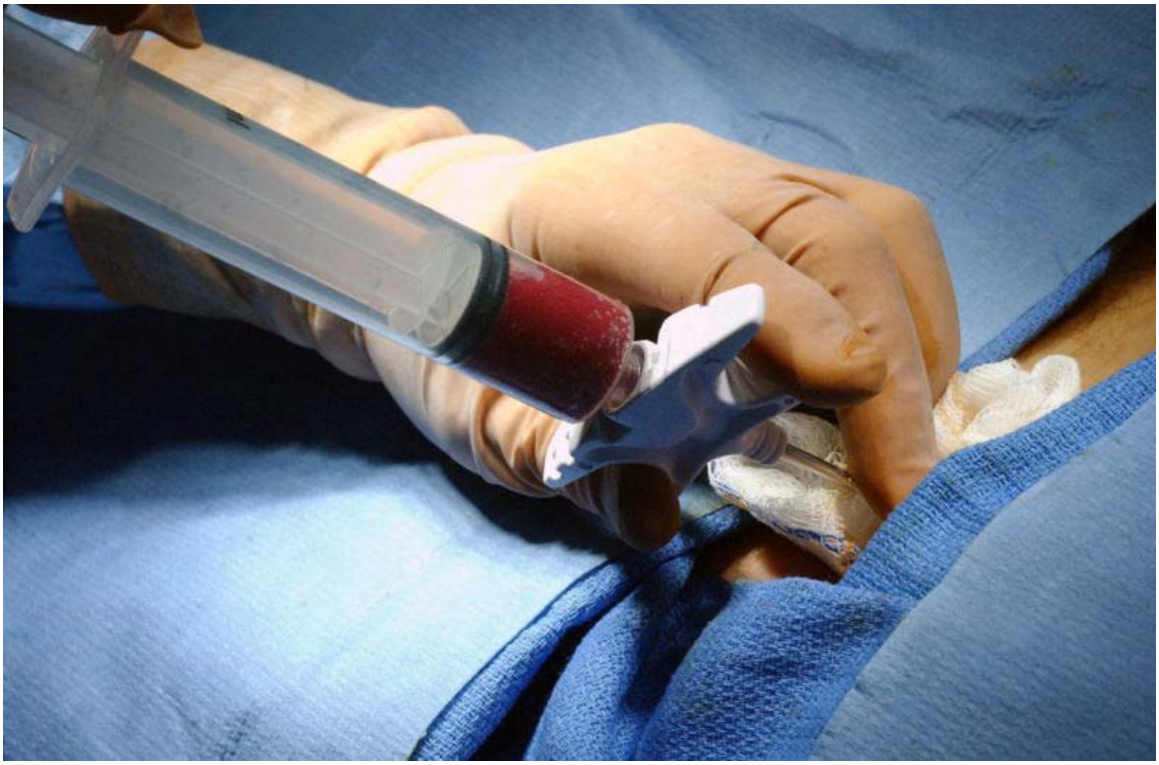
A compatible donor is found by doing additional HLA-testing from the blood of potential donors. The HLA genes fall in two categories (Type I and Type II). In general, mismatches of the Type-I genes (i.e. HLA-A, HLA-B, or HLA-C) increase the risk of graft rejection. A mismatch of an HLA Type II gene (i.e. HLA-DR, or HLA-DQB1) increases the risk of graft-versus-host disease. In addition a genetic mismatch as small as a single DNA base pair is significant so perfect matches require knowledge of the exact DNA sequence of these genes for both donor and recipient. Leading transplant centers currently perform testing for all five of these HLA genes before declaring that a donor and recipient are HLA-identical.

Race and ethnicity are known to play a major role in donor recruitment drives, as members of the same ethnic group are more likely to have matching genes, including the genes for HLA.

Sources and storage of cells

To limit the risks of transplanted stem cell rejection or of severe graft-versus-host disease in allogeneic HSCT, the donor should preferably have the same human leukocyte antigens (HLA) as the recipient. About 25 to 30 percent of allogeneic HSCT recipients have an HLA-identical sibling. Even so-called "perfect matches" may have mismatched minor alleles that contribute to graft-versus-host disease.

Bone marrow



Bone marrow harvest

In the case of a bone marrow transplant, the HSC are removed from a large bone of the donor, typically the pelvis, through a large needle that reaches the center of the bone. The technique is referred to as a bone marrow harvest and is performed under general anesthesia.

Peripheral blood stem cells

Peripheral blood stem cells are now the most common source of stem cells for allogeneic HSCT. They are collected from the blood through a process known as apheresis. The

donor's blood is withdrawn through a sterile needle in one arm and passed through a machine that removes white blood cells. The red blood cells are returned to the donor. The peripheral stem cell yield is boosted with daily subcutaneous injections of Granulocyte-colony stimulating factor, serving to mobilize stem cells from the donor's bone marrow into the peripheral circulation.

Amniotic fluid

It is also possible to extract hematopoietic stem cells from amniotic fluid for both autologous or heterologous use at the time of childbirth.

Umbilical cord blood

Umbilical cord blood is obtained when a mother donates her infant's umbilical cord and placenta after birth. Cord blood has a higher concentration of HSC than is normally found in adult blood. However, the small quantity of blood obtained from an umbilical cord (typically about 50 mL) makes it more suitable for transplantation into small children than into adults. Newer techniques using ex-vivo expansion of cord blood units or the use of two cord blood units from different donors are being explored to allow cord blood transplants to be used in adults.

It is used e.g. in children being born after preimplantation genetic diagnosis (PGD) for human leucocyte antigen (HLA) matching in order to donate to a sick sibling requiring HSCT.

Storage of HSC

Unlike other organs, bone marrow cells can be frozen for prolonged periods (cryopreserved) without damaging too many cells. This is necessary for autologous HSC because the cells must be harvested months in advance of the transplant treatment. In the case of allogeneic transplants fresh HSC are preferred in order to avoid cell loss that might occur during the freezing and thawing process. Allogeneic cord blood is stored frozen at a cord blood bank because it is only obtainable at the time of childbirth. To cryopreserve HSC a preservative, DMSO, must be added and the cells must be cooled very slowly in a control rate freezer to prevent osmotic cellular injury during ice crystal formation. HSC may be stored for years in a *cryofreezer* which typically utilizes liquid nitrogen because it is non-toxic and it is very cold (boiling point -196°C.)

Conditioning regimens

Myeloablative transplants

The chemotherapy or irradiation given immediately prior to a transplant is called the conditioning or preparative regimen, the purpose of which is to help eradicate the patient's disease prior to the infusion of HSC and to suppress immune reactions. The bone marrow can be *ablated* with dose-levels that cause minimal injury to other tissues. In

allogeneic transplants a combination of cyclophosphamide with busulfan or total body irradiation is commonly employed. This treatment also has an immunosuppressive effect which prevents rejection of the HSC by the recipient's immune system. The post-transplant prognosis often includes acute and chronic graft-versus-host disease which may be life-threatening; however in certain leukemias this can coincide with protection against cancer relapse owing to the *graft versus tumor* effect. *Autologous* transplants may also use similar conditioning regimens, but many other chemotherapy combinations can be used depending on the type of disease.

Non-myeloablative allogeneic transplants

This is a newer treatment approach using lower doses of chemotherapy and radiation which are too low to eradicate all of the bone marrow cells of a recipient. Instead, non-myeloablative transplants run lower risks of serious infections and transplant-related mortality while relying upon the *graft versus tumor* effect to resist the inherent increased risk of cancer relapse. Also significantly, while requiring high doses of immunosuppressive agents in the early stages of treatment, these doses are less than for conventional transplants. This leads to a state of mixed chimerism early after transplant where both recipient and donor HSC coexist in the bone marrow space.

Decreasing doses of immunosuppressive therapy then allows donor T-cells to eradicate the remaining recipient HSC and to induce the graft versus tumor effect. This effect is often accompanied by mild graft-versus-host disease, the appearance of which is often a surrogate for the emergence of the desirable graft versus tumor effect, and also serves as a signal to establish an appropriate dosage level for sustained treatment with low levels of immunosuppressive agents.

Because of their gentler conditioning regimens, these transplants are associated with a lower risk of transplant-related mortality and therefore allow patients who are considered too high-risk for conventional allogeneic HSCT to undergo potentially curative therapy for their disease. These new transplant strategies are still somewhat experimental, but are being used more widely on elderly patients unfit for myeloablative regimens and for whom the higher risk of cancer relapse may be acceptable.

Engraftment

After several weeks of growth in the bone marrow, expansion of HSC and their progeny is sufficient to normalize the blood cell counts and reinitiate the immune system. The offspring of donor-derived hematopoietic stem cells have been documented to populate many different organs of the recipient, including the heart, liver, and muscle, and these cells had been suggested to have the abilities of regenerating injured tissue in these organs. However, recent research has shown that such lineage infidelities does not occur as a normal phenomenon.

Complications

HSCT is associated with a high treatment-related mortality in the recipient (10% or higher), which limits its use to conditions that are themselves life-threatening. Major complications are veno-occlusive disease, mucositis, infections (sepsis) and graft-versus-host disease.

Infection

Bone marrow transplantation usually requires that the recipient's own bone marrow be destroyed ("myeloablation"). Prior to "engraftment" patients may go for several weeks without appreciable numbers of white blood cells to help fight infection. This puts a patient at high risk of infections, sepsis and septic shock, despite prophylactic antibiotics, and accounts for a large share of treatment-related mortality. The immunosuppressive agents employed in allogeneic transplants for the prevention or treatment of graft-versus-host disease further increase the risk of opportunistic infection. Immunosuppressive drugs are given for a minimum of 6-months after a transplantation, or much longer if required for the treatment of graft-versus-host disease. Transplant patients lose their acquired immunity, for example immunity to childhood diseases such as measles or polio. For this reason transplant patients must be re-vaccinated with childhood vaccines once they are off immunosuppressive medications.

Veno-occlusive disease

Severe liver injury is termed hepatic veno-occlusive disease (VOD). Elevated levels of bilirubin, hepatomegaly and fluid retention are clinical hallmarks of this condition. There is now a greater appreciation of the generalized cellular injury and obstruction in hepatic vein sinuses, and it has thus been referred to as sinusoidal obstruction syndrome (SOS). Severe cases are associated with a high mortality. Anticoagulants or defibrotide may be effective in reducing the severity of VOD but may also increase bleeding complications. Ursodiol has been shown to help prevent VOD, presumably by helping the flow of bile.

Mucositis

The injury of the mucosal lining of the mouth and throat and is a common regimen-related toxicity following ablative HSCT regimens. It is usually not life-threatening but is very painful, and prevents eating and drinking. Mucositis is treated with pain medications plus intravenous infusions to prevent dehydration and malnutrition.

Graft-versus-host disease

Graft-versus-host disease (GVHD) is an inflammatory disease that is unique to allogeneic transplantation. It is an attack of the "new" bone marrow's immune cells against the recipient's tissues. This can occur even if the donor and recipient are HLA-identical because the immune system can still recognize other differences between their tissues. It is aptly named graft-versus-host disease because bone marrow transplantation is the only

transplant procedure in which the transplanted cells must accept the body rather than the body accepting the new cells. *Acute graft-versus-host disease* typically occurs in the first 3 months after transplantation and may involve the skin, intestine, or the liver, and is often fatal. High-dose corticosteroids such as prednisone are a standard treatment; however this immuno-suppressive treatment often leads to deadly infections. *Chronic graft-versus-host disease* may also develop after allogeneic transplant. It is the major source of late treatment-related complications, although it less often results in death. In addition to inflammation, chronic graft-versus-host disease may lead to the development of fibrosis, or scar tissue, similar to scleroderma; it may cause functional disability and require prolonged immunosuppressive therapy. Graft-versus-host disease is usually mediated by T cells when they react to foreign peptides presented on the MHC of the host.

Graft-versus-tumor effect

Graft-versus-tumor effect (GVT) or "graft versus leukemia" effect is the beneficial aspect of the Graft-versus-Host phenomenon. For example, HSCT patients with either acute and in particular chronic graft-versus-host disease after an allogeneic transplant tend to have a lower risk of cancer relapse. This is due to a therapeutic immune reaction of the grafted donor T lymphocytes against the diseased bone marrow of the recipient. This lower rate of relapse accounts for the increased success rate of allogeneic transplants compared to transplants from identical twins, and indicates that allogeneic HSCT is a form of immunotherapy. GVT is the major benefit of transplants which do not employ the highest immuno-suppressive regimens.

Graft versus tumor is mainly beneficial in diseases with slow progress, e.g. chronic leukemia, low-grade lymphoma, and some cases multiple myeloma. However, it is less effective in rapidly growing acute leukemias.

If cancer relapses after HSCT, another transplant can be performed, infusing the patient with even more of the donor's white blood cells.

Prognosis

Prognosis in HSCT varies widely dependent upon disease type, stage, stem cell source, HLA-matched status (for allogeneic HCST) and conditioning regimen. A transplant offers a chance for cure or long-term remission if the inherent complications of graft versus host disease, immuno-suppressive treatments and the spectrum of opportunistic infections can be survived. In recent years, survival rates have been gradually improving across almost all populations and sub-populations receiving transplants.

Mortality for allogeneic stem cell transplantation can be estimated using the prediction model created by Sorror et al., using the Hematopoietic Cell Transplantation-Specific Comorbidity Index (HCT-CI). The HCT-CI was derived and validated by investigators at the Fred Hutchinson Cancer Research Center (Seattle, WA). The HCT-CI modifies and adds to a well-validated comorbidity index, the Charlson Comorbidity Index (CCI)

(Charlson et al.) The CCI was previously applied to patients undergoing allogeneic HCT but appears to provide less survival prediction and discrimination than the HCT-CI scoring system.

Risks to donor

The risks of a complication depend on patient characteristics, health care providers and the apheresis procedure, and the colony-stimulating factor used (G-CSF, GM-CSF). G-CSF drugs include Filgrastim (Neupogen, Neulasta), and lenograstim (Graslopin).

Drug risks

Filgrastim is typically dosed in the 10 microgram/kg level for 4–5 days during the harvesting of stem cells. The documented adverse effects of filgrastim include splenic rupture (indicated by left upper abdominal or shoulder pain, risk 1 in 40000), Adult respiratory distress syndrome (ARDS), alveolar hemorrage, and allergic reactions (usually expressed in first 30 minutes, risk 1 in 300). In addition, platelet and hemoglobin levels dip post-procedure, not returning to normal until one month.

The question of whether patients over 65 react the same as patients under 65 has not been sufficiently examined. Coagulation issues and inflammation of atherosclerotic plaques are known to occur as a result of G-CSF injection. G-CSF has also been described to induce genetic changes in mononuclear cells of normal donors. There is evidence that myelodysplasia (MDS) or acute myeloid leukaemia (AML)can be induced by GCSF in susceptible individuals.

Access risks

Blood was drawn peripherally in a majority of patients, but a central line to jugular/subclavian/femoral veins may be used in 16% of women and 4% of men. Adverse reactions during apheresis were experienced in 20% of women and 8% of men, these adverse events primarily consisted of numbness/tingling, multiple line attempts, and nausea.

Clinical observations

A study involving 2408 donors (18–60 years) indicated that bone pain (primarily back and hips) as a result of filgrastim treatment is observed in 80% of donors by day 4 post-injection. This pain responded to acetaminophen or ibuprofen in 65% of donors and was characterized as mild to moderate in 80% of donors and severe in 10%. Bone pain receded post-donation to 26% of patients 2 days post-donation, 6% of patients one week post-donation, and <2% 1 year post-donation. Donation is not recommended for those with a history of back pain. Other symptoms observed in more than 40% of donors include myalgia, headache, fatigue, and insomnia. These symptoms all returned to baseline 1 month post-donation, except for some cases of persistent fatigue in 3% of donors. . In one metastudy that incorporated data from 377 donors, 44% of patients

reported having adverse side effects after peripheral blood HSCT. Side effects included pain prior to the collection procedure as a result of GCSF injections, post-procedural generalized skeletal pain, fatigue and reduced energy.

Severe reactions

A study that surveyed 2408 donors found that serious adverse events (requiring prolonged hospitalization) occurred in 15 donors (at a rate of 0.6%), although none of these events were fatal. Donors were not observed to have higher than normal rates of cancer with up to 4–8 years of follow up. One study based on a survey of medical teams covered approximately 24,000 peripheral blood HSCT cases between 1993 and 2005, and found a serious cardiovascular adverse reaction rate of about 1 in 1500. This study reported a cardiovascular-related fatality risk within the first 30 days HSCT of about 2 in 10000. For this same group, severe cardiovascular events were observed with a rate of about 1 in 1500. The most common severe adverse reactions were pulmonary edema/deep vein thrombosis, splenic rupture, and myocardial infarction. Haematological malignancy induction was comparable to that observed in the general population with only 15 reported cases within 4 years.

Chapter 14

Splenectomy and Immunoelectrophoresis

Splenectomy

	Intervention: ***Splenectomy***
ICD-10 code:	
ICD-9 code:	41.43 41.5
MeSH	D013156
Other codes:	

A **splenectomy** is a surgical procedure that partially or completely removes the spleen.

Indications

The spleen, similar in structure to a large lymph node, acts as a blood filter. Current knowledge of its purpose includes the removal of old red blood cells and platelets, and the detection and fight against certain bacteria. It is also known to function as a site for the development of new red blood cells from their hematopoietic stem cell precursors, and particularly in situations in which the bone marrow, the normal site for this process, has been compromised by a disorder such as leukemia. The spleen is enlarged in a variety of conditions such as malaria, mononucleosis and most commonly in "cancers" of the lymphatics, such as lymphomas or leukemia.

It is removed under the following circumstances:

1. When it becomes very large such that it becomes destructive to platelets/red blood cells
2. For diagnosing certain lymphomas
3. Certain cases of wandering spleen
4. When platelets are destroyed in the spleen as a result of an auto-immune process
5. When the spleen bleeds following physical trauma
6. Following spontaneous rupture
7. For long-term treatment of congenital erythropoietic porphyria (CEP) if severe hemolytic anemia develops

8. The spread of gastric cancer to splenic tissue
9. When using the splenic artery for kidney revascularisation in renovascular hypertension.
10. For long-term treatment of congenital pyruvate kinase (PK) deficiency

The classical cause of traumatic damage to the spleen is a blow to the abdomen during a sporting event. In cases where the spleen is enlarged due to illness (mononucleosis), trivial activities, such as leaning over a counter or straining while defecating, can cause a rupture.

Procedure

Laparoscopy is the preferred procedure in cases where the spleen is not too large and when the procedure is elective. Open surgery is performed in trauma cases or if the spleen is enlarged. Either method is major surgery and is performed under general anesthesia. Vaccination for pneumococcus, *H. influenza* and meningococcus should be given pre-operatively if possible to minimize the chance of overwhelming post-splenectomy infection (OPSI), a rapid-developing and potentially fatal type of septicaemia. The spleen is located and disconnected from its arteries. The ligaments holding the spleen in place are dissected and the organ is removed. In some cases, one or more accessory spleens are discovered and also removed during surgery. The incisions are closed and when indicated, a drain is left. If necessary, tissue samples are sent to a laboratory for analysis.

Side effects

As splenectomy causes an increased risk of overwhelming sepsis due to encapsulated organisms (such as *S. pneumoniae* and *Haemophilus influenzae*) the patient should receive the pneumococcal conjugate vaccine (Prevnar), Hib vaccine, and the meningococcal vaccine. These bacteria often cause a sore throat under normal circumstances but after splenectomy, when infecting bacteria cannot be adequately opsonized, the infection becomes more severe.

An increase in blood leukocytes can occur following a splenectomy. The post-splenectomy platelet count may rise to abnormally high levels (thrombocytosis), leading to an increased risk of potentially fatal clot formation. There also is some conjecture that post-splenectomy patients may be at elevated risk of subsequently developing diabetes. Splenectomy may also lead to chronic neutrophilia. Splenectomy patients typically have Heinz bodies in their blood smears

Partial splenectomy

Much of the spleen's protective roles can be maintained if a small amount of spleen can be left behind. Where clinically appropriate, attempts are now often made to perform either surgical subtotal (partial) splenectomy, or partial splenic embolization. In particular, whilst vaccination and antibiotics provide good protection against the risks of

asplenia, this is not always available in poorer countries. However as it may take some time for the preserved splenic tissue to provide the full protection, it has been advised that preoperative vaccination still be given.

Immunoelectrophoresis

Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies reacting with the proteins to be separated or characterized. The methods were developed and used extensively during the second half of the 20th century. In somewhat chronological order: Immunoelectrophoretic analysis (one-dimensional immunoelectrophoresis *ad modum* Grabar), crossed immunoelectrophoresis (two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell), rocket-immunoelectrophoresis (one-dimensional quantitative immunoelectrophoresis *ad modum* Laurell), fused rocket immunoelectrophoresis *ad modum* Svendsen and Harboe, affinity immunoelectrophoresis *ad modum* Bøg-Hansen.

Agarose as 1 % gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, therefore immunoelectrophoresis allows characterization of enzyme activities and ligand binding etc in addition to electrophoretic separation.

The **immunoelectrophoretic analysis *ad modum* Grabar** is the classical method of immunoelectrophoresis. Proteins are separated by electrophoresis, then antibodies are applied in a trough next to the separated proteins and immunoprecipitates are formed after a period of diffusion of the separated proteins and antibodies against each other. The introduction of the immunoelectrophoretic analysis gave a great boost to protein chemistry, some of the very first results were the resolution of proteins in biological fluids and biological extracts. Among the important observations made were the great number of different proteins in serum, the existence of several immunoglobulin classes and their electrophoretic heterogeneity.

Crossed immunoelectrophoresis is also called two-dimensional quantitative immunoelectrophoresis *ad modum* Clarke and Freeman or *ad modum* Laurell. In this method the proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophorsis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. The sensitivity and resolving power of crossed immunoelectrophoresis is than that of the classical immunoelectrophoretic analysis and there are multiple variations of the technique useful for various purposes. Crossed immunoelectrophoresis has been used for studies of proteins in biological fluids, particularly human serum, and biological extracts.

Rocket immunoelectrophoresis is one-dimensional quantitative immunoelectrophoresis. The methods has been used for quantitation of human serum proteins before automated methods became available.

Fused rocket immunoelectrophoresis is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.

Affinity immunoelectrophoresis is based on changes in the electrophoretic pattern of proteins through biospecific interaction or complex formation with other macromolecules or ligands. Affinity immunoelectrophoresis has been used for estimation of binding constants, as for instance with lectins or for characterization of proteins with specific features like glycan content or ligand binding. Some variants of affinity immunoelectrophoresis are similar to affinity chromatography by use of immobilized ligands.

The open structure of the immunoprecipitate in the agarose gel will allow additional binding of radioactively labeled antibodies to reveal specific proteins. This variation has been used for identification of allergens through reaction with IgE.

Two factors determine that immunoelectrophoretic methods are not widely used. First they are rather work intensive and require some manual expertise. Second they require rather large amounts of polyclonal antibodies. Today gel electrophoresis followed by electroblotting is the preferred method for protein characterization because its ease of operation, its high sensitivity, and its low requirement for specific antibodies. In addition proteins are separated by gel electrophoresis on the basis of their apparent molecular weight, which is not accomplished by immunoelectrophoresis, but nevertheless immunoelectrophoretic methods are still useful when non-reducing conditions are needed.

Chapter 15

RAST Test and Coombs Test

RAST test

A **RAST test** (short for **radioallergosorbent test**) is a blood test used to determine to what substances a person is allergic. This is different from a skin allergy test, which determines allergy by the reaction of a person's skin to different substances.

Because there are other tests that help with confirmation, results are best interpreted by a doctor.

Indication

The RAST test is an alternative to skin tests to elucidate the causal allergen to an allergy.

Advantages of the RAST test range from: excellent reproducibility across the full measuring range of the calibration curve, but decreased sensitivity and specificity when compared with skin prick testing. In general, this method of blood testing (in-vitro, out of body) vs skin-prick testing (in-vivo, in body) has a major advantage: it is not always necessary to remove the patient from an anthihistamine medication regimen, and if the skin conditions (such as eczema) are so widespread that allergy skin testing cannot be done.

Still, when possible, allergy skin testing is the preferred method in comparison with various *in vitro* tests for assessing the presence of specific IgE antibodies because it is more sensitive and specific, simpler to use, and less expensive.

Method

The RAST test is a radioimmunoassay test to detect specific IgE antibodies to suspected or known allergens. IgE is the antibody associated with Type I allergic response: for example, if a person exhibits a high level of IgE directed against pollen, the test may indicate the person is allergic to pollen (or pollen-like) proteins. A person who has outgrown an allergy may still have a positive IgE years after exposure.

The suspected allergen is bound to an insoluble material and the patient's serum is added. If the serum contains antibodies to the allergen, those antibodies will bind to the allergen. Radiolabeled anti-human IgE antibody is added where it binds to those IgE antibodies already bound to the insoluble material. The unbound anti-human IgE antibodies are washed away. The amount of radioactivity is proportional to the serum IgE for the allergen.

History

The market-leading RAST methodology was invented and marketed in 1974 by Pharmacia Diagnostics AB, Uppsala, Sweden, and the acronym RAST is actually a brand name. In 1989, Pharmacia Diagnostics AB replaced it with a superior test named the ImmunoCAP Specific IgE blood test, which literature may also describe as: CAP RAST, CAP FEIA (fluorenzymeimmunoassay), and Pharmacia CAP. A review of applicable quality assessment programs shows that this new test has replaced the original RAST in approximately 80% of the world's commercial clinical laboratories, where specific IgE testing is performed. The newest version, the ImmunoCAP Specific IgE 0-100, is the only specific IgE assay to receive FDA approval to quantitatively report to its detection limit of 0.1kU/l. This clearance is based on the CLSI/NCCLS-17A Limits of Detection and Limits of Quantitation, October 2004 guideline.

RAST often are used to test for allergies when:
• a physician advises against the discontinuation of medications that can interfere with test results or cause medical complications;
• a patient suffers from severe skin conditions such as widespread eczema or psoriasis; or
• a patient has such a high sensitivity level to suspected allergens that any administration of those allergens might result in potentially serious side effects.

Scale

The RAST test is scored on a scale from 0 to 6:

RAST rating	IgE level (KU/L)	comment
0	< 0.35	ABSENT OR UNDETECTABLE ALLERGEN SPECIFIC IgE
1	0.35 - 0.69	LOW LEVEL OF ALLERGEN SPECIFIC IgE
2	0.70 - 3.49	MODERATE LEVEL OF ALLERGEN SPECIFIC IgE
3	3.50 - 17.49	HIGH LEVEL OF ALLERGEN SPECIFIC IgE
4	17.50 - 49.99	VERY HIGH LEVEL OF ALLERGEN SPECIFIC IgE
5	50.0 - 100.00	VERY HIGH LEVEL OF ALLERGEN SPECIFIC IgE

6	> 100.00	EXTREMELY HIGH LEVEL OF ALLERGEN SPECIFIC IgE

Coombs test

Coombs test (also known as **Coombs' test**, **antiglobulin test** or **AGT**) refers to two clinical blood tests used in immunohematology and immunology. The two Coombs tests are the **direct Coombs test** (also known as **direct antiglobulin test** or **DAT**), and the **indirect Coombs test** (also known as **indirect antiglobulin test** or **IAT**).

The more commonly used test, the Direct Coombs test, is used to test for autoimmune hemolytic anemia.

In certain diseases or conditions an individual's blood may contain IgG antibodies that can specifically bind to antigens on the red blood cell (RBC) surface membrane, and their circulating red blood cells (RBCs) can become coated with IgG alloantibodies and/or IgG autoantibodies. Complement proteins may subsequently bind to the bound antibodies. The **direct Coombs test** is used to detect these antibodies or complement proteins that are bound to the surface of red blood cells; a blood sample is taken and the RBCs are washed (removing the patient's own plasma) and then incubated with antihuman globulin (also known as "Coombs reagent"). If this produces agglutination of RBCs, the direct Coombs test is positive, a visual indication that antibodies (and/or complement proteins) are bound to the surface of red blood cells.

The **indirect Coombs test** is used in prenatal testing of pregnant women, and in testing blood prior to a blood transfusion. It detects antibodies against RBCs that are present unbound in the patient's serum. In this case, serum is extracted from the blood, and the serum is incubated with RBCs of known antigenicity. If agglutination occurs, the indirect Coombs test is positive.

Mechanism

The two Coombs tests are based on the fact that anti-human antibodies, which are produced by immunizing non-human species with human serum, will bind to human antibodies, commonly IgG or IgM. Animal anti-human antibodies will also bind to human antibodies that may be fixed onto antigens on the surface of red blood cells (also referred to as RBCs), and in the appropriate test tube conditions this can lead to agglutination of RBCs. The phenomenon of agglutination of RBCs is important here, because the resulting clumping of RBCs can be visualised; when clumping is seen the test is positive and when clumping is not seen the test is negative.

Common clinical uses of the Coombs test include the preparation of blood for transfusion in cross-matching, screening for atypical antibodies in the blood plasma of pregnant

women as part of antenatal care, and detection of antibodies for the diagnosis of immune-mediated haemolytic anemias.

Coombs tests are done on serum from venous blood samples which are taken from patients by venepuncture. The venous blood is taken to a laboratory (or blood bank), where trained scientific technical staff do the Coombs tests. The clinical significance of the result is assessed by the physician who requested the Coombs test, perhaps with assistance from a laboratory-based hematologist.

Direct Coombs test

The direct Coombs test (also known as the **direct antiglobulin test** or DAT) is used to detect if antibodies or complement system factors have bound to RBC surface antigens *in vivo*. The DAT is not currently required for pre-transfusion testing but may be included by some laboratories.

Examples of diseases that give a positive direct Coombs test

The direct Coombs test is used clinically when immune-mediated hemolytic anemia (antibody-mediated destruction of RBCs) is suspected. A positive Coombs test indicates that an immune mechanism is attacking the patient's own RBC's. This mechanism could be autoimmunity, alloimmunity or a drug-induced immune-mediated mechanism.

Examples of alloimmune hemolysis

- Hemolytic disease of the newborn (also known as HDN or erythroblastosis fetalis)
    - Rh D hemolytic disease of the newborn (also known as Rh disease)
    - ABO hemolytic disease of the newborn (the indirect Coombs test may only be weakly positive)
    - Anti-Kell hemolytic disease of the newborn
    - Rh c hemolytic disease of the newborn
    - Rh E hemolytic disease of the newborn
    - Other blood group incompatibility (RhC, Rhe, Kidd, Duffy, MN, P and others)
- Alloimmune hemolytic transfusion reactions

Examples of autoimmune hemolysis

- Warm antibody autoimmune hemolytic anemia
    - Idiopathic
    - Systemic lupus erythematosus
    - Evans' syndrome (antiplatelet antibodies and hemolytic antibodies)

- Cold antibody autoimmune hemolytic anemia
    - Idiopathic cold hemagglutinin syndrome

- Infectious mononucleosis
- Paroxysmal cold hemoglobinuria (rare)

Drug-induced immune-mediated hemolysis

- Methyldopa (IgG mediated type II hypersensitivity)
- Penicillin (high dose)
- Quinidine (IgM mediated activation of classical complement pathway and Membrane attack complex, MAC)

(A memory device to remember that the *DAT* tests the RBCs and is used to test infants for *haemolytic disease of the newborn* is: ***R**h **D**isease*; ***R*** = RBCs, ***D*** = DAT.)

Laboratory method

The patient's red blood cells (RBCs) are washed (removing the patient's own serum) and then incubated with antihuman globulin (also known as Coombs reagent). If immunoglobulin or complement factors have been fixed on to the RBC surface in-vivo, the antihuman globulin will agglutinate the RBCs and the direct Coombs test will be positive. (A visual representation of a positive direct Coombs test is shown in the upper half of the schematic).

Indirect Coombs test

The indirect Coombs test (also known as the **indirect antiglobulin test** or IAT) is used to detect in-vitro antibody-antigen reactions. It is used to detect very low concentrations of antibodies present in a patient's plasma/serum prior to a blood transfusion. In antenatal care, the IAT is used to screen pregnant women for antibodies that may cause hemolytic disease of the newborn. The IAT can also be used for compatibility testing, antibody identification, RBC phenotyping, and titration studies.

Examples of clinical uses of the indirect Coombs test

Blood transfusion preparation

The indirect Coombs test is used to screen for antibodies in the preparation of blood for blood transfusion. The donor's and recipient's blood must be ABO and Rh D compatible. Donor blood for transfusion is also screened for infections in separate processes.

- Antibody screening

A blood sample from the recipient and a blood sample from every unit of donor blood are screened for antibodies with the indirect Coombs test. Each sample is incubated against a wide range of RBCs that together exhibit a full range of surface antigens (i.e. blood types).

- Cross matching

The indirect Coombs test is used to test a sample of the recipient's serum against a sample of the blood donor's RBCs. This is sometimes called cross-matching blood.

Antenatal antibody screening

The indirect Coombs test is used to screen pregnant women for IgG antibodies that are likely to pass through the placenta into the fetal blood and cause haemolytic disease of the newborn.

Laboratory method

The IAT is a two-stage test. (A cross match is shown visually in the lower half of the schematic as an example of an indirect Coombs test).

First stage

Washed test red blood cells (RBCs) are incubated with a test serum. If the serum contains antibodies to antigens on the RBC surface, the antibodies will bind onto the surface of the RBCs.

Second stage

The RBCs are washed three or four times with isotonic saline and then incubated with antihuman globulin. If antibodies have bound to RBC surface antigens in the first stage, RBCs will agglutinate when incubated with the antihuman globulin (also known Coombs reagent) in this stage, and the indirect Coombs test will be positive.

Titrations

By diluting a serum containing antibodies the quantity of the antibody in the serum can be gauged. This is done by using doubling dilutions of the serum and finding the maximum dilution of test serum that is able to produce agglutination of relevant RBCs.

Coombs reagent

Coombs reagent (also known as **Coombs antiglobulin** or **antihuman globulin**) is used in both the direct Coombs test and the indirect Coombs test. Coombs reagent is antihuman globulin. It is made by injecting human globulin into animals, which produce polyclonal antibodies specific for human immunoglobulins and human complement system factors. More specific Coombs reagents or monoclonal antibodies can be used.

Enhancement media

Both IgM and IgG antibodies bind strongly with their antigens. IgG antibodies are most reactive at 37°C. IgM antibodies are easily detected in saline at room temperature as IgM antibodies are able to bridge between RBC's owing to their large size, efficiently creating what is seen as agglutination. IgG antibodies are smaller and require assistance to bridge well enough to form a visual agglutination reaction. Reagents used to enhance IgG detection are referred to as potentiators. RBCs have a net negative charge called zeta potential which causes them to have a natural repulsion for one another. Potentiators reduce the zeta potential of RBC membranes. Common potentiators include low ionic strength solution (LISS), albumin, polyethylene glycol (PEG), and proteolytic enzymes.

History of the Coombs test

The Coombs test was first described in 1945 by Cambridge immunologists Robin Coombs (after whom it is named), Arthur Mourant and Rob Race. Historically, it was done in test tubes. Today, it is commonly done using microarray and gel technology.

Chapter 16

Patch Test (Medicine)

Eczema can be produced by exogenous factors and is referred to as exogenous or contact dermatitis. There are two forms of contact eczema: irritant and allergic. Irritant dermatitis occurs after chronic exposure to mild irritants (e.g soaps, detergents in a wet environment). "Handwashing" eczema is an example of irritant dermatitis produced by chronic exposure to soaps and detergents since rarely these products produce allergic reactions. Allergic contact dermatitis, on the other hand, is the manifestation of an acquired allergic response to chemicals that normally do not produce irritant and toxic effects. Since allergic contact dermatitis occur in a small percentage of the population, one individual might be the only one affected in the environment, even though other people are also exposed to the chemical(s). To develop an allergic reactions there had to be previous exposure(s) to the incriminated chemical(s). The longer one is exposed to some of these chemicals, the greater the chances of becoming allergic, and once one become allergic, subsequent exposure will be followed by more severe eczematous reactions. In the case of allergic contact dermatitis, high concentrations or prolonged exposure to the chemicals are not prerequisite to induce a reaction as is the case with an irritant contact dermatitis. In other words, casual exposure to the chemical(s) could be sufficient to induce a skin reaction if you have an allergic contact dermatitis. A **patch test** is a method used to determine if a specific substance causes allergic inflammation of the skin. Any individual with eczema suspected of having allergic contact dermatitis and/or atopic dermatitis needs patch testing.

Patch Testing helps identify which substances may be causing a reaction in a patient. It is intended to produce a local allergic reaction on a small area of your back where the diluted chemicals were planted. The chemicals included in the patch test kit are the offenders in approximately 85-90 percent of contact allergic eczema and include chemicals present in metals (e.g. nickel), rubber, leather, hair dyes, formaldehyde, lanolin, fragrance, preservative and other additives.

Mechanism

A patch test relies on the principle of a type IV hypersensitivity reaction.

The first step in becoming allergic is sensitization. When the skin is exposed to an allergen, the antigen presenting cells (APCs) - also known as Langerhans cell or Dermal

Dendritic Cell - eat up substance (phagocytoze) and break it into smaller pieces. This is where a substance is recognized by immune cells in the skin. They then put parts of the substance onto their surface (technically holds the part of the molecule on the surface in the major histocompatibility complex type two (MHC-II) . Once this is done the APC moves down the lymphatic system to a lymph node where it presents this part of the substance (what we now call an antigen) to an particular immune cell called a CD4+ T-cell or T-helper cell. The T-cell, if it recognizes the substance as dangerous, expands in number and sends out more of itself to the skin, at the site of antigen exposure. When the skin is again exposed to the antigen, the memory t-cells in the skin recognize the antigen and produce cytokines (chemical signals) which cause more T-cells to migrate from blood vessels. This starts a complex immune cascade leading to skin inflammation, itching and the typical rash of contact dermatitis. In general, it takes 2 to 4 days for a response in patch testing to develop. The patch test is really just induction of a contact dermatitis in a small area.

Interestingly, the size of the molecule necessary to be picked up and recognized is ten times the size of the largest molecule that can pass through the skin. Therefore, it is likely that an antigen (like Nickel) when it has passed through the skin, combines with something else before it is recognized.

Process

Prior to testing, avoid taking oral prednisone or other immunosuppressive medications for at least a week prior to testing. Steroid inhalers are OK to use. Avoid sunlight/sunburn for at least a week on the back as this may suppress positive reactions. Antihistamines such as diphenhydramine (Benadryl) or cetirizine (Zyrtec) are permissible prior to and during testing.

Application of the patch tests will take about half an hour, though many times the overall appointment time will be longer as your provider will take an extensive history. Tiny quantities of 25 to ~150 materials (allergens) in individual square plastic or round aluminium chambers are applied to the upper back. They are kept in place with special hypoallergenic adhesive tape. The patches stay in place undisturbed for at least 48 hours. Getting the back wet during patch testing should be avoided (no shower). Vigorous exercise or stretching may disrupt the tests.

At the second appointment, usually 48 hours later, the patches will be removed. Sometimes further patches are applied. The back is marked with an indelible black felt tip pen or other suitable marker to identify the test sites and a preliminary reading is done. These marks must be visible at the third appointment, usually 24-48 hours later (72-96 hours after application). The back should be checked and if necessary re-marked on several occasions between the 2nd and 3rd appointments. In some cases, a reading at 7 days may be requested, especially if a special metal series is tested.

Interpretation of the results

The dermatologist or allergist will complete a record form at the second and third appointments (usually 48 and 72/96 hour readings). The result for each test site is recorded. One system used is as follows:

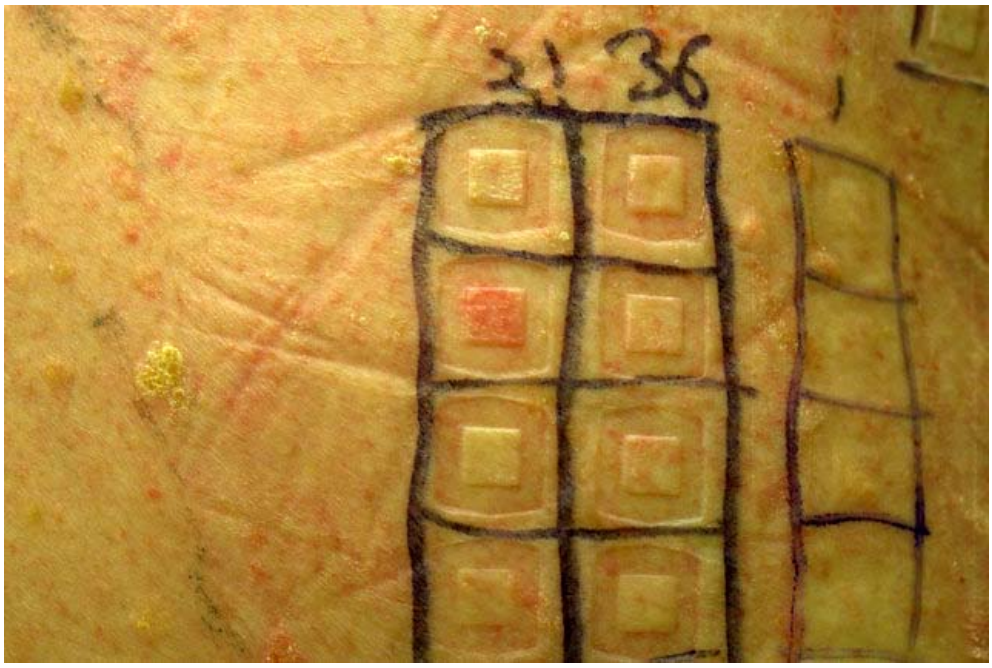
Negative (-)

Irritant reaction (IR)

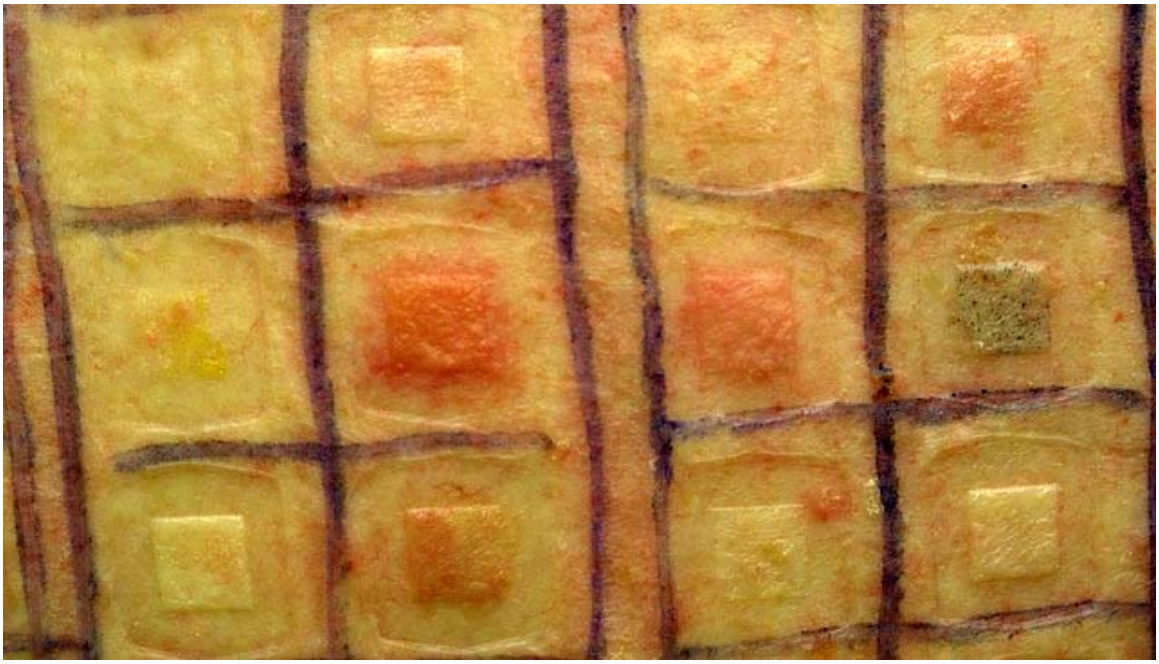
Equivocal / uncertain (+/-)

Weak positive

(+)



Strong positive (++)



Extreme reaction (+++)

Irritant reactions include miliaria (sweat rash), follicular pustules and burn-like reactions. Uncertain reactions refer to a pink area under the test chamber. Weak positives are slightly elevated pink or red plaques, usually with mild vesiculation. Strong positives are ‘papulovesicles’ and extreme reactions have spreading redness, severe itching and blisters or ulcers.

Relevance is determined by exposure to the positive allergen(s) and is rated as definite, probable, possible, past or unknown. For an allergen to have definite relevance, the product the patient is exposed to must be tested and also be positive in addition to the test allergen. Probable would be used to describe an positive allergen ingredient which is in a product the patient uses (i.e. quaternium-15 listed in a moisturizing cream used on the sites of dermatitis). The interpretation of the results requires considerable experience and training. A positive patch test(s), might not explain the present skin problem since the test only indicates that the individual became allergic during the encounters with that chemical(s) at some point in their life. Relevance, therefore, has to be established by determining the casual relationship between the positive test(s) and the eczema. The confirmation of relevance will occur after the patient has avoided exposure to the chemical(s) and after they have noticed that the improvement or clearance of your dermatitis is directly related to this avoidance. This outcome usually occurs within four to six weeks after stopping the exposure to the chemical(s).

If all patch tests are negative, the eczema is probably not due to an allergic reaction to a contactant. It is possible, however, that you were not tested to other chemical(s) that can produce allergic reactions on the rare occasions. If the suspicion is high in spite of negative patch testing, further investigation might be required. This can be discussed during the final evaluation of the patch test procedure.

Common allergens

The most frequent allergen that was recorded in many research studies all around the world is Nickel. Nickel allergy is more prevalent in young women and it is especially associated with ear piercing or any nickel-containing watch, belt, zipper or jewelry. Other common allergens are surveyed in North America by the North American Contact Dermatitis Group (NACDG).

The latest update of top allergens from 2005-2006 were: Nickel sulfate (19.0%), Myroxylon pereirae (balsam of Peru, 11.9%), fragrance mix I (11.5%), quaternium-15 (10.3%), neomycin (10.0%), bacitracin (9.2%), formaldehyde (9.0%), cobalt chloride (8.4%), methyldibromoglutaronitrile/phenoxyethanol (5.8%), p-phenylenediamine (5.0%), potassium dichromate (4.8%), carba mix (3.9%), thiuram mix (3.9%), diazolidinyl urea (3.7%), and 2-bromo-2-nitropropane-1,3-diol (3.4%)

Food Allergy

There is often an assumption that certain foods can cause or worsen skin complaints like eczema. While it is true that food allergies exist, there is very little evidence that cutting out foods such as milk and eggs actually improves eczema.

Dermatologists may refer patients with suspected food allergies for patch testing. Sometimes this is justified as certain food additives and flavorings can cause dermatitis around the mouth, around the anus and vagina as food allergens pass out of the body or cause a widespread rash on the skin. While this is controversial, allergens such as nickel, Balsam of Peru, parabens, sodium benzoate or cinnamic aldehyde may worsen or cause skin rashes.

However, the foods that cause urticaria (hives) or anaphylaxis (such as peanuts) cause a type I hypersensitivity reaction whereby the part of the food molecule is directly recognized by cells close to the skin called mast cells. Mast cells have antibodies on their surface called immunoglobulin E (IgE). These act as receptors and if they recognize the allergen, they release their contents, causing an immediate allergic reaction. Type I reactions like anaphylaxis are immediate and do not take 2 to 4 days to appear. In a recent study of patients with chronic hives who were patch tested, those who were found allergic and avoided all contact with their allergen, including dietary intake, stopped having hives. Those who started eating their allergen again had recurrence of their hives. Often, patch testing for food allergies is not necessary, but in selected individuals it may be helpful.

Chapter 17

Complement Fixation Test and Radioimmunoassay

Complement fixation test

The **complement fixation test** is an immunological medical test that can be used to detect the presence of either specific antibody or specific antigen in a patient's serum. It was widely used to diagnose infections, particularly with microbes that are not easily detected by culture methods, and in rheumatic diseases. However, in clinical diagnostics labs it has been largely superseded by other serological methods such as ELISA and by DNA-based methods of pathogen detection, particularly PCR.

Process

The complement system is a system of serum proteins that react with antigen-antibody complexes. If this reaction occurs on a cell surface, it will result in the formation of trans-membrane pores and therefore destruction of the cell. The basic steps of a complement fixation test are as follows:

1. Serum is isolated from the patient.
2. Patients naturally have different levels of complement proteins in their serum. To negate any effects this might have on the test, the complement proteins in the patient's serum must be destroyed and replaced by a known amount of standardized complement proteins.
    1. The serum is heated in such a way that all of the complement proteins--but none of the antibodies-- within it are destroyed. (This is possible because complement proteins are much more susceptible to destruction by heat than antibodies.)
    2. A known amount of standard complement proteins are added to the serum. (These proteins are frequently obtained from guinea pig serum.)
3. The antigen of interest is added to the serum.
4. Sheep red blood cells (sRBCs) which have been pre-bound to anti-sRBC antibodies are added to the serum. The test is considered negative if the solution turns pink at this point and positive otherwise.

If the patient's serum contains antibodies against the antigen of interest, they will bind to the antigen in step 3 to form antigen-antibody complexes. The complement proteins will react with these complexes and be depleted. Thus when the sRBC-antibody complexes are added in step 4, there will be no complement left in the serum. However, if no antibodies against the antigen of interest are present, the complement will not be depleted and it will react with the sRBC-antibody complexes added in step 4, lysing the sRBCs and spilling their contents into the solution, thereby turning the solution pink.

Testing for antigen

While detection of antibodies is the more common test format, it is equally possible to test for the presence of antigen. In this case, the patient's serum is supplemented with specific antibody to induce formation of complexes; addition of complement and indicator sRBC is performed as before.

Quantitative testing

The test can be made quantitative by setting up a series of dilutions of patient serum and determining the highest dilution factor that will still yield a positive CF test. This dilution factor corresponds to the titer.

Radioimmunoassay

Radioimmunoassay (RIA), an *in vitro* nuclear medicine, is a very sensitive technique used to measure concentrations of antigens (for example, hormone levels in the blood) by use of antibodies. As such, it can be seen as the inverse of a radiobinding assay, which quantifies an antibody by use of corresponding antigens.

Although the RIA technique is extremely sensitive and extremely specific, it requires specialized equipment, but remains the least expensive method to perform such tests. It requires special precautions and licensing, since radioactive substances are used. Today it has been supplanted by the ELISA method, where the antigen-antibody reaction is measured using colorimetric signals instead of a radioactive signal. However, because of its robustness, consistent results and low price per test , RIA methods are again becoming popular. It is generally more simple to perform than a bioassay

The RAST test (radioallergosorbent test) is an example of radioimmunoassay. It is used to detect the causative allergen for an allergy.

Method

To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine attached to tyrosine. This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured using a gamma counter. Using known standards, a binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.

History

It was developed by Rosalyn Yalow and Solomon Aaron Berson in the 1950s. In 1977, Rosalyn Sussman Yalow received the Nobel Prize in Medicine for the development of the RIA for insulin: the precise measurement of minute amounts of such a hormone was considered a breakthrough in endocrinology.

With this technique, separating bound from unbound antigen is crucial. Initially, the method of separation employed was the use of a second "anti-antibody" directed against the first for precipitation and centrifugation. The use of charcoal suspension for precipitation was extended but replaced later by Drs. Werner and Acebedo at Columbia University for RIA of T3 and T4. An ultramicro RIA for human TSH was published in BBRC (1975) by Drs. Acebedo, Hayek et al.

Chapter 18

Retroperitoneal Lymph Node Dissection and Chromatin Immunoprecipitation

Retroperitoneal lymph node dissection

Retroperitoneal lymph node dissection, commonly referred to as RPLND, is a procedure to remove abdominal lymph nodes to treat testicular cancer, as well as help establish its exact stage and type. It is usually performed using an incision that extends from the sternum to several inches below the navel. While laparoscopic methods may be used, they have been considered less effective by some surgeons.

Testicular cancer spreads in a well-known pattern, and the lymph nodes in the retroperitoneum are a primary landing site during spread of the disease. Examining the removed lymphatic tissue will determine the extent of spread of any malignant disease and if no malignant tissue is found, the cancer may be more accurately considered as a stage I cancer, limited to the testis.

The procedure is becoming standard treatment for clinical stage I and II non-seminomatous germ cell tumors (NSGCTT) because of the low mortality and relapse rate with this procedure, as compared with the alternative, which is observation. Also, NSGCTT is considered more aggressive than seminomas, the "other" kind of testicular cancer. Seminomas are also much more sensitive to radiation than NSGCTT's, so the noninvasive radiation treatment is often preferred over RPLND.

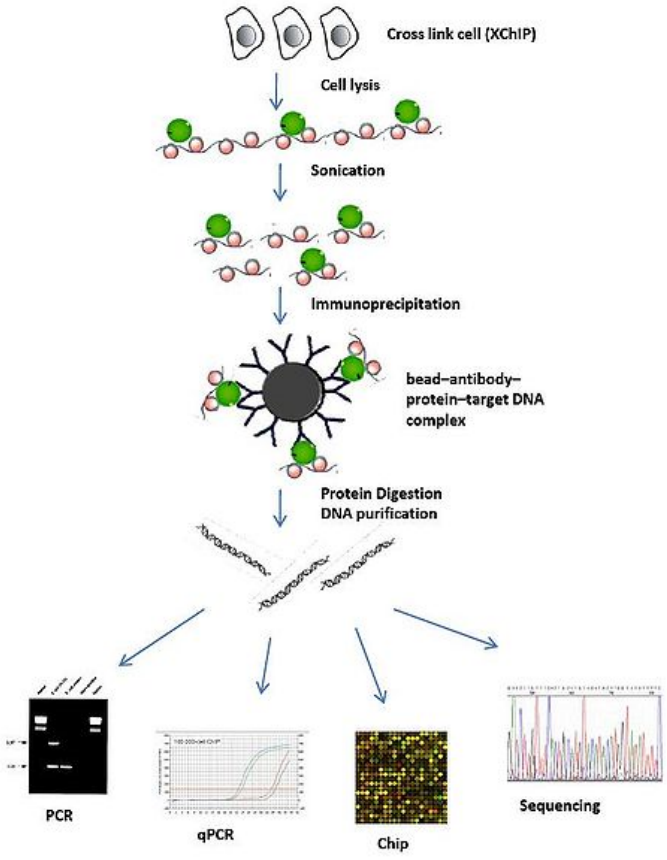
The potential problems in RPLND have mostly to do with nerves: sympathetic nerves running parallel to the spinal cord may be damaged or severed during the procedure, which can result in infertility, an inability to ejaculate, or the inability to have an erection. This is why most often, a nerve-sparing technique is used where possible. A less invasive form using laparoscopic techniques (L-RPLND) exists, which is more costly, time-consuming, and requires special equipment that not every hospital may have. Open RPLND (O-RPLND), which is performed by opening the abdomen to get inside, has more room for problems, but is an equally effective way to remove the lymph nodes. Disadvantages of an open RPLND include longer recovery time, sometimes with physiotherapy required to help the patient regain the ability to walk after being bed-

bound. As with any major surgery, infection is a possibility, and bowel obstructions and adhesions are another possible side effect.

There are different schools of thought about the need to perform RPLND after orchiectomy, and it depends on the type of tumour, and what stage it is in. Most American Doctors recommend surgery, whereas in Europe, chemotherapy is more often used. An RPLND may be performed to remove non-malignant tumour remnants which persist after chemotherapy; without further treatment these may once more become malignant, and may be resistant to the combination of chemotherapy previously used.

Chemotherapy before RPLND is considered an effective approach, because it is possible that it suffices and no relapse occurs. However, in the event that the cancer does recur, chemotherapy can complicate surgery.

Chromatin immunoprecipitation



The procedure of chromatin immunoprecipitation (ChIP) assay and methods of analysis

Chromatin Immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to investigate the interaction between proteins and DNA in the cell. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoters or other DNA binding sites, and possibly defining cistromes. ChIP also aims to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifiers.

Briefly, the method is as follows: protein and associated chromatin in a cell lysate are temporarily bonded, the DNA-protein complexes (chromatin-protein) are then sheared and DNA fragments associated with the protein(s) of interest are selectively immunoprecipitated, then the associated DNA fragments are purified and their sequence is determined. These DNA sequences are supposed to be associated with the protein of interest *in vivo*.

Typical ChIP

Technically, there are mainly two types of ChIP, primarily differing in the starting chromatin preparation. The first uses reversibly cross-linked chromatin sheared by sonication called cross-linked ChIP (XChIP). Native ChIP (NChIP) uses native chromatin sheared by micrococcal nuclease digestion,

Cross-linked ChIP (XChIP)

Cross-linked ChIP is mainly suited for mapping DNA target of transcription factors or other chromatin-associated proteins, by using reversibly cross-linked chromatin as starting material. The agent for reversible cross-link could be formaldehyde or UV light. Then the cross-linked chromatin are usually sheared by sonication, providing fragments of 300-1000 base pairs (bp) in length. Mild formaldehyde crosslinking followed by nuclease digestion has been used to shear the chromatin. Chromatin fragments of 400-500bp have proven to be suitable for ChIP assays as they cover two to three nucleosomes.

Cell debris in the sheared lysate is then cleared by sedimentation and protein–DNA complexes are selectively immunoprecipitated using specific antibodies to the protein(s) of interest. The antibodies are commonly coupled to agarose, sepharose or magnetic beads. The immunoprecipitated complexes (i.e., the bead–antibody–protein–target DNA sequence complex) are then collected and washed to remove non-specifically bound chromatin, the protein-DNA cross-link is reversed and proteins are removed by digestion with proteinase K.

The DNA associated with the complex is then purified and identified by polymerase chain reaction (PCR), microarrays (ChIP-on-chip), molecular cloning and sequencing, or direct high-throughput sequencing (ChIP-seq).

Native ChIP (NChIP)

Native ChIP is mainly suited for mapping the DNA target of histone modifiers. Generally, native chromatin is used as starting chromatin. As histones wrap around DNA to form nucleosomes, they are naturally linked. Then the chromatin is sheared by micrococcal nuclease digestion, which cuts DNA at the length of the linker, leaving nucleosomes intact and providing DNA fragments of one nucleosome (200bp) to five nucleosomes (1000bp) in length.

Thereafter, methods similar to XChIP are used for clearing the cell debris, immunoprecipitating the protein of interest, removing protein from the immunoprecipated complex, and purifying and analyzing the complex-associated DNA.

Comparison of XChIP and NChIP

The major advantage for NChIP is antibody specificity. It is important to note that most antibodies to modified histones are raised against unfixed, synthetic peptide antigens and that the epitopes they need to recognize in the XChIP may be disrupted or destroyed by formaldehyde cross-linking, particularly as the cross-links are likely to involve lysine e-amino groups in the N-terminals, disrupting the epitopes. This is likely to explain the consistently low efficiency of XChIP protocols compare to NChIP.

But XChIP and NChIP have different aims and advantage against each other, XChIP is for mapping target site of transcription factors and other chromatin associated proteins, NChIP is for mapping the target site of histone modifiers (see Table 1).

Table 1 Advantages and disadvantages of NChIP and XChIP

	XChIP	NChIP
Advantages	Suitable for transcriptional factors, or any other weakly binding chromatin associated proteins. Applicable to any organisms where native protein is hard to prepare	Testable antibody specificity Better antibody specificity as target protein naturally intact Better chromatin and protein recovery efficiency due to Better antibody specificity
Disadvantages	Inefficient chromatin recovery due to antibody target protein epitope disruption May cause false positive result due to fixation of transient proteins to chromatin Wide range of chromatin shearing size due to random cut by sonication.	Usually not suitable for non-histone proteins Nucleosomes may rearrange during digestion

History and New ChIP methods

XChIP was pioneered by Alexander Varshavsky and co-workers in the 1980s, and has been extensively developed and refined. NChIP approach was first described by Hebbes *et al*., 1988, and also been developed and refined quickly. The typical ChIP assay usually take 4–5 days, and require 10^6~ 10^7 cells at least. Now new techniques on ChIP could be achieved as few as 100~1000 cells and complete within one day.

- **Carrier ChIP** (CChIP): This approach could use as few as 100 cells by adding *Drosophila* cells as carrier chromatin to reduce loss and facilitate precipitation of the target chromatin. However, it demands highly specific primers for detection of the target cell chromatin from the foreign carrier chromatin background, and it takes two to three days.

- **Fast ChIP** (qChIP): The fast ChIP assay reduced the time by shortening two steps in a typical ChIP assay: *(i)* an ultrasonic bath accelerates the rate of antibody binding to target proteins—and thereby reduces immunoprecipitation time *(ii)* a resin-based (Chelex-100) DNA isolation procedure reduces the time of cross-link reversal and DNA isolation. However, the fast protocol is suitable only for large cell samples (in the range of 10^6~10^7). Up to 24 sheared chromatin samples can be processed to yield PCR-ready DNA in 5 hours, allowing multiple chromatin factors be probed simultaneously and/or looking at genomic events over several time points.

- **Quick and quantitative ChIP** (Q^2ChIP) : The assay uses 100,000 cells as starting material and is suitable for up to 1,000 histone ChIPs or 100 transcription factor ChIPs. Thus many chromatin samples can be prepared in parallel and stored, and Q^2ChIP can be undertaken in a day .

- **MicroChIP** (μChIP): chromatin is usually prepared from 1,000 cells and up to 8 ChIPs can be done in parallel without carriers. The assay can also start with 100 cells, but only suit for one ChIP. It can also use small (1 mm^3) tissue biopsies and microChIP can be done within one day.

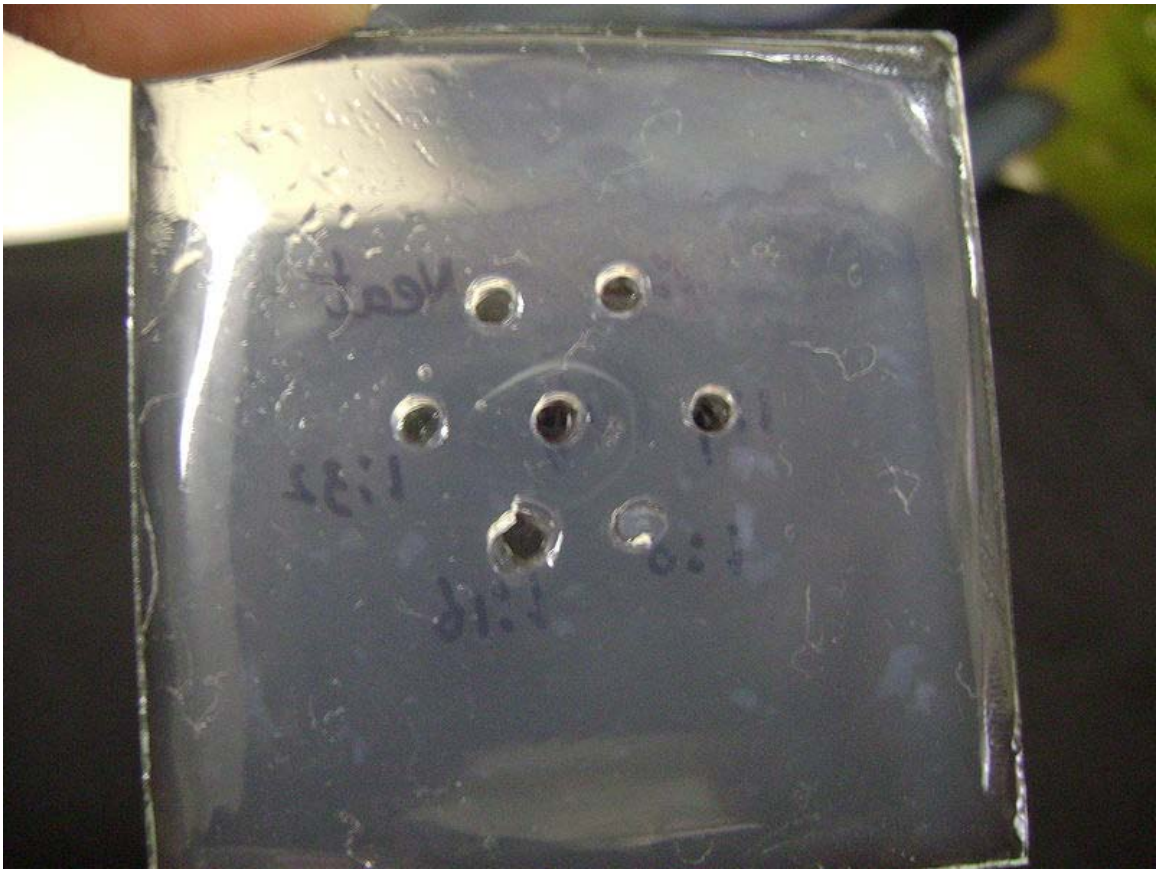
- **Matrix ChIP**: This is a microplate-based ChIP assay with increased throughput and simplified the procedure. All steps are done in microplate wells without sample transfers, enabling a potential for automation. It enables 96 ChIP assays for histone and various DNA-bound proteins in a single day .

ChIP could also been applied for genome wide analysis when combined with microarray technology (ChIP-on-chip) and second generation DNA-sequencing technology (Chip-Sequencing). ChIP can also combine with paired-end tags sequencing in Chromatin Interaction Analysis using Paired End Tag sequencing (ChIA-PET), a technique developed for large-scale, de novo analysis of higher-order chromatin structures.

Chapter 19

Ouchterlony Double Immunodiffusion, ELISA and Radial Immunodiffusion

Ouchterlony double immunodiffusion



Picture of an Ouchterlony double immunodiffusion plate, after immunodiffusion has taken place. In this, titre value of an antigen is quantified. The central well has an antibody, and the surrounding wells have decreasing concentration of the corresponding antigen

Ouchterlony double immunodiffusion (also known as **agar gel immunodiffusion** or **passive double immunodiffusion**) is a simple, rather dated method which is still considered to be the gold standard for detection of extractable nuclear antigens (ENAs).

Procedure

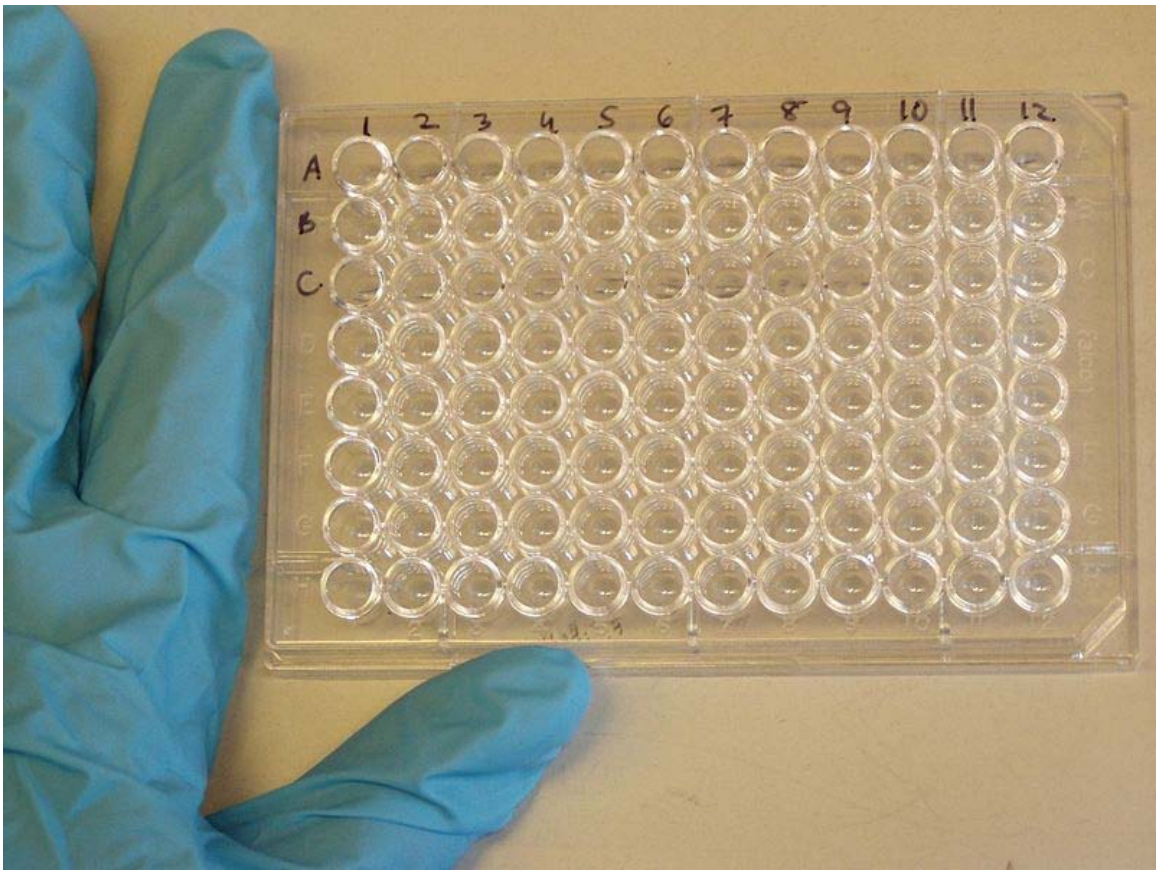
A gel plate is cut to form a series of holes ("wells") in the gel. A sample extract of interest (for example human cells harvested from tonsil tissue) is placed in one well, and sera or purified antibodies are placed in another well and the plate left for 48 hours to develop. During this time the antigens in the sample extract and the antibodies each diffuse out of their respective wells. Where the two diffusion fronts meet, if any of the antibodies recognize any of the antigens, they will bind to the antigens and form what is known as an immune complex. This immune complex precipitates in the gel to give a thin white line, which is a visual signature of antigen recognition.

The method can be conducted in parallel with multiple wells filled with different antigen mixtures and multiple wells with different antibodies or mixtures of antibodies, and antigen-antibody reactivity can be seen by observing between which wells the precipitate is observed. When more than one well is used there are many possible outcomes based on the reactivity of the antigen and antibody selected. The zone of equivalence lines may give a full identity (i.e. a continuous line), partial identity (i.e. a continuous line with a spur at one end), or a non-identity (i.e. the two lines cross completely).

Theory

Precipitation occurs with most antigens because the antigen is multivalent (i.e. has several antigenic determinants per molecule to which antibodies can bind). Antibodies have at least two antigen binding sites (and in the case of IgM there is a multimeric complex with up to 10 antigen binding sites), thus large aggregates or gel-like lattices of antigen and antibody are formed. Experimentally, an increasing amount of antigen is added to a constant amount of antibody in solution, initially at low antigen concentration, all of the antigen is contained in the precipitate. This is called the antibody-excess zone (i.e. prozone phenomenon). As more antigen is added, the amount protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the zone of equivalence or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone.

ELISA



A 96-well microtiter plate being used for ELISA

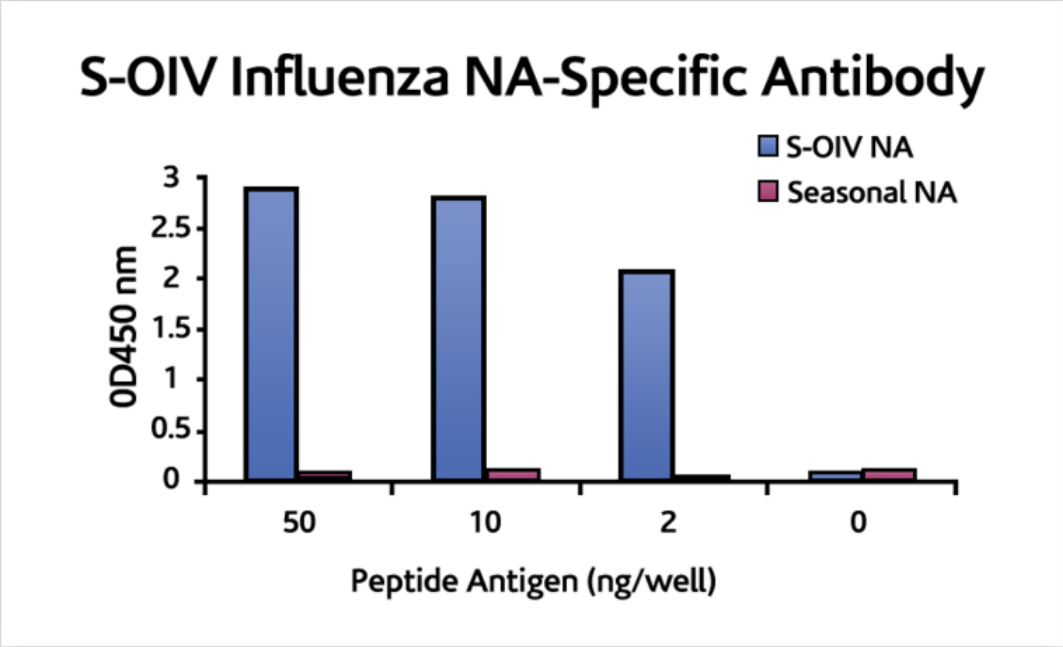
Enzyme-linked immunosorbent assay (**ELISA**), also known as an **enzyme immunoassay** (**EIA**), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries. In simple terms, in ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal, most commonly a colour change in a chemical substrate.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an

enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Traditional ELISA typically involves chromogenic reporters and substrates that produce some kind of observable color change to indicate the presence of antigen or analyte. Newer ELISA-like techniques utilize fluorogenic, electrochemiluminescent, and real-time PCR reporters to create quantifiable signals. These new reporters can have various advantages including higher sensitivities and multiplexing. In technical terms, newer assays of this type are not strictly ELISAs, as they are not "enzyme-linked" but are instead linked to some non-enzymatic reporter. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

Applications



ELISA results using S-OIV A neuraminidase antibody at 1 µg/ml to probe the immunogenic and the corresponding seasonal influenza A neuraminidase peptides at 50, 10, 2, and 0 ng/ml.

Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations (such as with the HIV test or West Nile Virus). It has also found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds, and eggs. ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.

The ELISA was the first screening test widely used for HIV because of its high sensitivity. In an ELISA, a person's serum is diluted 400-fold and applied to a plate to which HIV antigens are attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to other antibodies — is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and a negative result.

A cut-off point may be determined by comparing it with a known standard. If an ELISA test is used for drug screening at workplace, a cut-off concentration, 50 ng/mL, for example, is established, and a sample that contains the standard concentration of analyte will be prepared. Unknowns that generate a signal that is stronger than the known sample are "positive." Those that generate weaker signal are "negative."

History

Before the development of the ELISA, the only option for conducting an immunoassay was radioimmunoassay, a technique using radioactively-labeled antigens or antibodies. In radioimmunoassay, the radioactivity provides the signal, which indicates whether a specific antigen or antibody is present in the sample. Radioimmunoassay was first described in a paper by Rosalyn Sussman Yalow and Solomon Berson published in 1960.

Because radioactivity poses a potential health threat, a safer alternative was sought. A suitable alternative to radioimmunoassay would substitute a non-radioactive signal in place of the radioactive signal. When enzymes (such as peroxidase) react with appropriate substrates (such as ABTS or 3,3’,5,5’-Tetramethylbenzidine), a change in color occurs, which is used as a signal. However, the signal has to be associated with the presence of antibody or antigen, which is why the enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and G.B. Pierce. Since it is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container; i.e., the *immunosorbent* has to be prepared. A technique to accomplish this was published by Wide and Jerker Porath in 1966.

In 1971, Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in The Netherlands independently published papers that synthesized this knowledge into methods to perform EIA/ELISA.

Types

"Indirect" ELISA

The steps of "indirect" ELISA follows the mechanism below:-

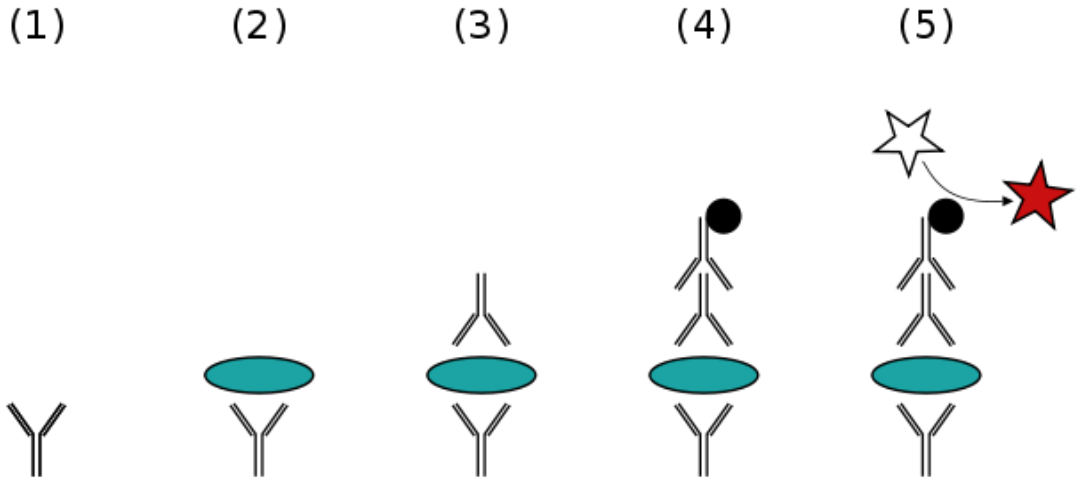
- A buffered solution of the antigen to be tested for is added to each well of a microtiter plate, where it is given time to adhere to the plastic through charge interactions.
- A solution of non-reacting protein, such as bovine serum albumin or casein, is added to block any plastic surface in the well that remains uncoated by the antigen.
- Next the primary antibody is added, which binds specifically to the test antigen that is coating the well. This primary antibody could also be in the serum of a donor to be tested for reactivity towards the antigen.
- Afterwards, a secondary antibody is added, which will bind the primary antibody. This secondary antibody often has an enzyme attached to it, which has a negligible effect on the binding properties of the antibody.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme. The color change shows that secondary antibody has bound to primary antibody, which strongly implies that the donor has had an immune reaction to the test antigen. This can be helpful in a clinical setting, and in R&D.
- The higher the concentration of the primary antibody that was present in the serum, the stronger the color change. Often a spectrometer is used to give quantitative values for color strength.

The enzyme acts as an amplifier; even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules. Within common-sense limitations, the enzyme can go on producing color indefinitely, but the more primary antibody is present in the donor serum the more secondary antibody + enzyme will bind, and the faster color will develop. A major disadvantage of the indirect ELISA is that the method of antigen immobilization is non-specific; when serum is used as the source of test antigen, all proteins in the sample may stick to the microtiter plate well, so small concentrations of analyte in serum must compete with other serum proteins when binding to the well surface. The sandwich or direct ELISA provides a solution to this problem, by using a "capture" antibody specific for the test antigen to pull it out of the serum's molecular mixture.

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result (yes or no) for a sample. The cutoff between positive and negative is determined by the analyst and may be statistical. Two or three times the standard deviation (error inherent in a test) is often used to distinguish positive from negative samples. In quantitative ELISA, the optical density (OD) of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule. For example, if a test sample returns an OD

of 1.0, the point on the standard curve that gave OD = 1.0 must be of the same analyte concentration as your sample.

Sandwich ELISA



A sandwich *ELISA*. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

A less-common variant of this technique, called "sandwich" ELISA, is used to detect sample antigen. The steps are as follows:

1. Prepare a surface to which a known quantity of capture antibody is bound.
2. Block any nonspecific binding sites on the surface.
3. Apply the antigen-containing sample to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. Apply enzyme linked primary antibodies as detection antibodies that also bind specifically to the antigen.
6. Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
7. Apply a chemical that is converted by the enzyme into a color or fluorescent or electrochemical signal.
8. Measure the absorbency or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

The image to the right includes the use of a secondary antibody conjugated to an enzyme, though, in the technical sense, this is not necessary if the primary antibody is conjugated to an enzyme. However, use of a secondary-antibody conjugate avoids the expensive process of creating enzyme-linked antibodies for every antigen one might want to detect. By using an enzyme-linked antibody that binds the Fc region of other antibodies, this same enzyme-linked antibody can be used in a variety of situations. Without the first

layer of "capture" antibody, any proteins in the sample (including serum proteins) may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized. Use of the purified specific antibody to attach the antigen to the plastic eliminates a need to purify the antigen from complicated mixtures before the measurement, simplifying the assay, and increasing the specificity and the sensitivity of the assay.

Competitive ELISA

A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different than the first two examples:

1. Unlabeled antibody is incubated in the presence of its antigen (Sample).
2. These bound antibody/antigen complexes are then added to an antigen-coated well.
3. The plate is washed, so that unbound antibody is removed. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
4. The secondary antibody, specific to the primary antibody is added. This second antibody is coupled to the enzyme.
5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.

For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present.

(Note that some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with your sample antigen (unlabeled). The more antigen in the sample the less labeled antigen is retained in the well and the weaker the signal).

It is common that the antigen is not first positioned in the well.

Multiple and Portable ELISA (M&P ELISA)(ELISA Reverse in published papers)

A new technique (EP 1 499 894 B1 in EPO Bulletin 25.02.209 N. 2009/09; USPTO 7510687 in USPTO Bulletin 31.03.2009; ZL 03810029.0 in SIPO PRC Bulletin 08.04.2009) uses a solid phase made up of an immunosorbent polystyrene rod with 8-12 protruding ogives. The entire device is immersed in a test tube containing the collected sample and the following steps (washing, incubation in conjugate and incubation in chromogenous) are carried out by dipping the ogives in microwells of standard microplates pre-filled with reagents.

The advantages of this technique are as follows:

1. The ogives can each be sensitized to a different reagent, allowing the simultaneous detection of different antibodies and/or different antigens for multi-target assays
2. The sample volume can be increased to improve the test sensitivity in clinical (saliva, urine), food (bulk milk, pooled eggs) and environmental (water) samples
3. One ogive is left unsensitized to measure the non-specific reactions of the sample
4. The use of laboratory supplies for dispensing sample aliquots, washing solution and reagents in microwells is not required, facilitating the deveopment of ready-to-use lab-kits and on-site kits.

Radial immunodiffusion

Radial immunodiffusion (or Mancini method, Mancini immunodiffusion, single radial immunodiffusion assay) is an immunodiffusion technique used in immunology to determine the quantity of an antigen by measuring the diameters of circles of precipitin complexes surrounding samples of the antigen that mark the boundary between the antigen and an antibody suspended in a medium, such as an agar gel. The diameters of the circles increase with time as the antigen diffuses into the medium, reacts with the antibody, and forms insoluble precipitin complexes.

Antigen-antibody complexes are small and soluble when in antigen excess. Therefore, precipitation near the center of the circle is usually less dense than it is near the circle's outer edge, where antigen is less concentrated.

The quantity and concentration of insoluble antigen-antibody complexes at the outer edge of the circle increases with time. Therefore, the clarity and density of the outer edge increases with time.

Expansion of the circle reaches an end point and stops when antigen and antibody reach equivalence. However, the clarity and density of the outer edge may continue to increase after the circle stops expanding.

For most antigens, the area and the square of the diameter of the circle at the circle's end point are directly proportional to the quantity of antigen and are inversely proportional to the concentration of antibody. Therefore, a graph that compares the quantities or concentrations of antigen in the original samples with the areas or the squares of the diameters of the precipitin circles on linear scales will usually be a straight line when all circles have reached their end points. Circles created by small quantities of antigen reach their end points before large quantities do. Therefore, if areas or diameters of circles are measured while some, but not all, circles have stopped expanding, such a graph will be

straight in the portion that contains the smaller quantities or concentrations of antigen and will be curved in the portion that contains the larger quantities or concentrations.

While circles are still expanding, a graph that compares the quantities or concentrations of the antigen on a logarithmic scale with the diameters or areas of the circles on a linear scale may be a straight line. However, circles of the precipitate are smaller and less distinct during expansion than they are after expansion has ended. Further, temperature affects the rate of expansion, but does not affect the size of a circle at its end point. In addition, the range of circle diameters for the same quantities or concentrations of antigen is smaller while some circles are enlarging than they are after all circles have reached their end points. Therefore, measurements of the sizes of circles and of graphs produced from such measurements are often less accurate when circles are expanding than they are after expansion has ended. For that reason, it is often more desirable to take measurements after all circles have reached their end points than it is to take measurements while some or all circles are still expanding.

Measurements of large circles are more accurate than are those of small circles. It is therefore often desirable to adjust the concentration of antibody and the quantity of antigen to assure that precipitin rings will be large.

Chapter 20

Enzyme Multiplied Immunoassay Technique and Immunofluorescence

Enzyme multiplied immunoassay technique

Enzyme multiplied immunoassay technique, or **EMIT**, is a common method for screening urine and blood for drugs, both legal or illicit. First introduced by Syva Company in 1973, it is the first homogeneous immunoassay to be widely used commercially.

A mix and read protocol has been developed that is exceptionally simple and rapid. The most widely used applications for EMIT are for therapeutic drug monitoring (serum) and as a primary screen for abused drugs and their metabolites (urine). The US patents covering the major aspects of the method, 3,817,837 and 3,875,011, have expired. While still sold by Siemens Healthcare under its original trade name, EMIT, assay kits with different names that employ the same technology are supplied by other companies. The test is not particularly accurate, especially with regard to test results for cannabis. When the Food and Drug Administration approved EMIT, it did so with the strict provision that positive test results should be confirmed by an alternative testing method.

Technique

1) Combine a sample containing an unknown concentration of antigen (Ag) & a solution containing a known concentration of antibody against the Ag (Ab).

2) Allow binding of Ag & Ab (incubation #1).

3) Add a known concentration of prepared Ag-enzyme conjugate.

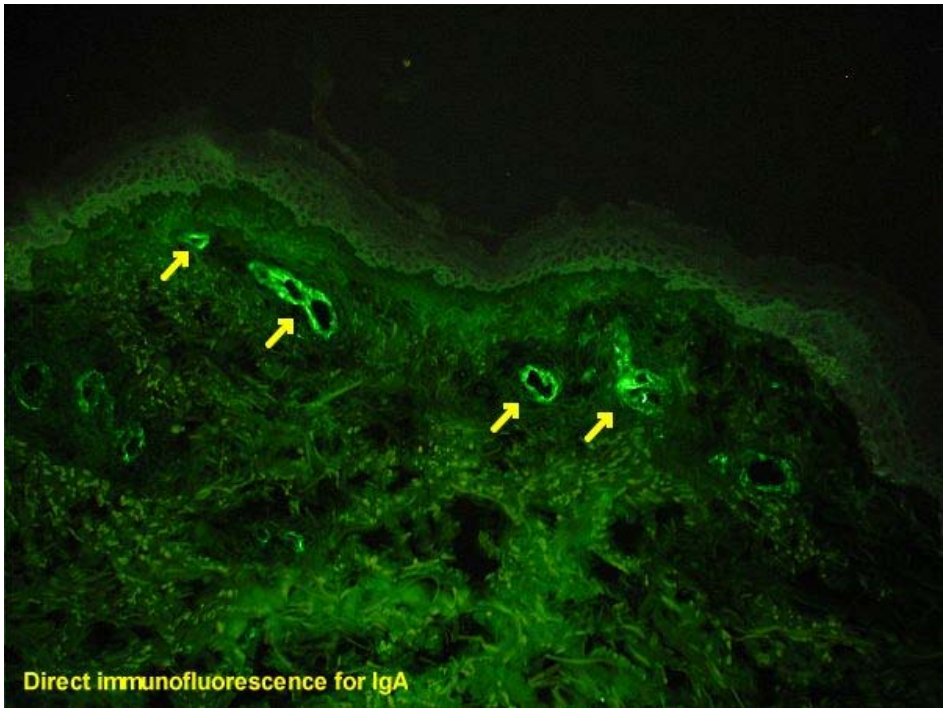
4) Allow binding of Ag-enzyme conjugate with any remaining unbound Ab in solution (incubation #2).

The conjugate is constitutionally active; binding to any unbound Ab will render it inactive.

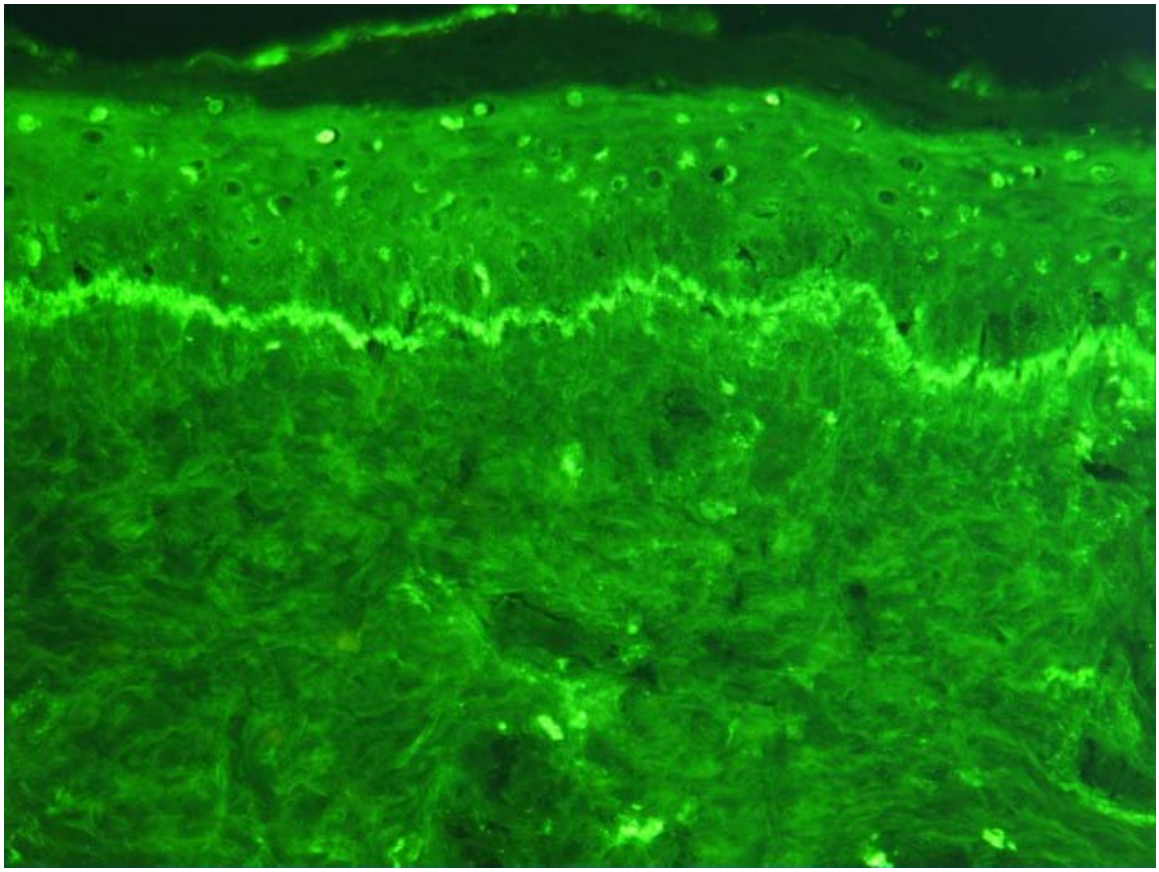
5) Add enzyme substrate.

6) Measure enzyme activity.

Immunofluorescence



Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgA antibody. The skin is from a patient with Henoch-Schonlein purpura: IgA deposits are found in the walls of small superficial capillaries (yellow arrows). The pale wavy green area on top is the epidermis, the bottom fibrous area is the dermis.



Microphotograph of a histological section of human skin prepared for **direct immunofluorescence** using an anti-IgG antibody. The skin is from a patient with systemic lupus erythematosus and shows IgG deposit at two different places: The first is a band-like deposit along the epidermal basement membrane ("lupus band test" is positive). The second is within the nuclei of the epidermal cells (anti-nuclear antibodies).

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on biological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualisation of the distribution of the target molecule through the sample. Immunofluorescence is a widely used example of immunostaining and is a specific example of immunohistochemistry that makes use of fluorophores to visualise the location of the antibodies.

Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyse the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluoresence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA. Several microscope designs can be used for analysis of immunofluorescence samples; the simplest is the epifluorescence microscope, and the confocal microscope is also widely used. Various super-resolution microscope designs that are capable of much higher resolution can also be used.

Types of immunofluorescence

There are two classes of immunofluorescence techniques, primary (or direct) and secondary (or indirect).

Primary (direct)

Primary, or direct, immunofluorescence uses a single antibody that is chemically linked to a fluorophore. The antibody recognises the target molecule and binds to it, and the fluorophore it carries can be detected via microscope. This technique has several advantages over the secondary (or indirect) protocol below because of the direct conjugation of the antibody to the fluorophore. This reduces the number of steps in the staining procedure, is therefore faster, and can avoid some issues with antibody cross-reactivity or non-specificity, which can lead to increased background signal.

Secondary (indirect)

Secondary, or indirect, immunofluorescence uses two antibodies; the first (the primary antibody) recognises the target molecule and binds to it, and the second (the secondary antibody), which carries the fluorophore, recognises the primary antibody and binds to it. This protocol is more complex than the primary (or direct) protocol above and takes more time but allows more flexibility.

This protocol is possible because an antibody consists of two parts, a variable region (which recognizes the antigen) and an invariant region (which makes up the structure of the antibody molecule). A researcher can generate several primary antibodies that recognize various antigens (have different variable regions), but all share the same invariant region. All these antibodies may therefore be recognized by a single secondary antibody. This saves the cost of modifying the primary antibodies to directly carry a fluorophore.

Different primary antibodies with different invariant regions are typically generated by raising the antibody in different species. For example, a researcher might create primary antibodies in a goat that recognize several antigens, and then employ dye-coupled rabbit secondary antibodies that recognize the goat antibody invariant region ("rabbit anti-goat" antibodies). The researcher may then create a second set of primary antibodies in a mouse that could be recognised by a separate "donkey anti-mouse" secondary antibody. This allows re-use of the difficult-to-make dye-coupled antibodies in multiple experiments.

Limitations

As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g., Alexa Fluors, Seta Fluors, or DyLight Fluors).

Immunofluorescence is only limited to fixed (i.e., dead) cells when structures within the cell are to be visualized because antibodies cannot cross the cell membrane. Proteins in the supernatant or on the outside of the cell membrane can be bound by the antibodies, this allows for living cells can be stained. Depending on the fixative that is being used, proteins of interest might become cross-linked and this could result in either false positive or false negative signals due to unspecific binding.

An alternative approach is using recombinant proteins containing fluorescent protein domains, e.g., green fluorescent protein (GFP). Use of such "tagged" proteins allows determination of their localization in live cells. Even though this seems to be an elegant alternative to immunofluorescence, the cells have to be transfected or transduced with the GFP-tag, and as a consequence they become at least S1 or above organisms that require stricter security standards in a laboratory.