Teacher Background

Using ELISA to Measure Concentrations of Sex Hormones over the Menstrual Cycle in Females and Over Lifespan in Males

Note: The Teacher Background Section is meant to provide information for the teacher about the topic and is tied very closely to the PowerPoint slide show. For greater understanding, the teacher may want to play the slide show as he/she reads the background section. For the students, the slide show can be used in its entirety or can be edited as necessary for a given class.

What Is ELISA?

ELISA is an acronym for Enzyme-Linked ImmunoSorbent Assay. It is a biochemical assay widely used to detect the presence of and quantity of a particular protein in a sample, such as hormones, antibodies, and bacteria or viruses. The assay uses the coupling of antigens and antibodies and relies on the specificity (the ability to discriminate among diverse proteins) and affinity (the ability to tightly bind to molecules) of antibodies for antigens. Using the ELISA method, one can determine how much antibody is present in a sample by starting with an antigen, or one can determine how much antigen is present in a sample by starting with the antibody. (1, 2, 3, 12)

What Are Antigens?

Antigens are any foreign substance in the body. Antigens include "not-self" molecules like foreign proteins, viruses, and environmental pollutants and other foreign substances like asbestos, tattoo ink, and cigarette smoke. Antigens also include "not-self" cells like bacteria, parasites (Protista, Fungi, Plantae, and Animalia viruses, and environmental pollutants and other foreign substances like asbestos, tattoo ink, and cigarette smoke. cells), foreign transplanted tissue, and cancerous cells, (3, 13, 14)

What Are Antibodies and How Are They Produced?

Antibodies are large glycoprotein molecules produced by B-lymphocytes during the humoral immune response to antigens introduced into the body. Both B-lymphocytes (B-cells) and T-lymphocytes (T-cells) are white blood cells which form from hematopoietic (blood) stem cells in the bone marrow. B-cells mature in the bone marrow and form the humoral (antibody-mediated) immune system while T-cells migrate to and mature in the thymus gland and form the cellular (cell-mediated) immune system.

How Do B-Cells and T-Cells Work Together to Fight Infection?

B-cells and T-cells work together in the lymphatic system, especially in the lymph nodes, to fight off invasion and infection from antigens. B-lymphocytes mainly work by producing antibodies in response to antigens and then marking those antigens to be destroyed by the T-cells. Each B-cell can make its own distinct antibody in response to the antigen in contact with it and each antibody is designed to bind to a specific surface binding site or epitope on the antigen. There are millions of different types of antibodies circulating in an individual's bloodstream and they are based on exposure to antigens in his/her environment. Over 80% of the human antibodies are in the immunoglobulin class IgG. IgG antibodies are shaped like a Y and are found in the blood, lymph, and intestine. These proteins have a molecular weight of 150,000 Daltons and are made of 2 long (heavy) chains coded from DNA on chromosome 14, and 2 short (light) chains coded from DNA on either chromosomes 2 or 22. These are all connected by disulphide bonds. Most of the molecule is composed of a region that remains the same from one IgG molecule to another (called the constant region). However, the ends of the forked part of the Y are variable and this accounts for how each IgG molecule is able to bind only to a specific antigen. (3, 4)

When Were Antibody-Antigen Interactions Discovered?

An understanding of antibody-antigen interactions first began in 1890 when Emil von Behring from Germany and Shibashuro Kitasato from Japan were working with toxins produced by diphtheria bacillus. They noticed than an "antitoxin" formed in the blood of animals infected with this disease and that the antitoxin serum from those infected animals could be successfully transferred to other animals that had been given a lethal dose of toxin and the animals survived. With their toxin-antitoxin theory, they proposed the humoral theory of immunity against bacterial infection. In 1901, Emil von Behring was the first to be awarded the Nobel Prize in Physiology or Medicine "for his work on serum therapy, especially its application against diphtheria". It is unclear why Shibashuro Kitasato was not recognized since they worked together on the antitoxin experiments.

In an article written in 1891, Paul Erlich first used the term "antikorper", the German word for antibody and in 1897, he proposed the idea that the "side chain" receptors on the surface of cells could bind to specific toxins in a "lock-and-key" interaction. Paul Erlich shared the 1908 Nobel Prize in Physiology or Medicine with Ilya Mechnikov, a Russian scientist "in recognition for their work on immunity".

In 1940, Linus Pauling at California Institute of Technology confirmed the lock-and-key theory proposed by Erlich and was awarded the Nobel Prize in Chemistry in 1954 "for his research into the nature of the chemical bond and its application to the elucidation of the structure of complex substances", including antibodies and the nature of serological reactions. In 1948, Astrid Fagreaus, Karolinska Institutet, Stockholm, Sweden, presented evidence that B-lymphocytes in the form of plasma cells formed the antibodies circulating in the bloodstream.

By the 1960s, Gerald Edelman at Rockefeller University, New York, and Rodney Porter at the University of Oxford, England, worked out the structure and complete amino acid sequence of the antibody IgG, and jointly shared the 1972 Nobel Prize in Physiology or Medicine "for their discoveries concerning the chemical structure of antibodies". (3, 5, 6, 7, 8, 9)

What Are RadioimmunoAssays and How Were They Developed?

RadioimmunoAssays were developed in 1960 and opened the door for the development of ELISA tests in 1971. Based on their understanding of the specificity and affinity of the antigen-antibody interaction, Solomon Berson and Rosalyn Yalow at the Veterans Administration Hospital in New York in 1960 developed a method called RadioImmunoAssay (RIA) and used it to measure the amount of endogenous plasma insulin by tagging the insulin with a radioactive label. Originally, iodine-131 (half-life = 8.1 days, beta and gamma emitter) was used for RadioimmunoAssay but it created potential health risks for those running the assays. Later, Iodine-125 (half-life = 60.14 days, weak gamma emitter) was marketed with equal but safer results.

Rosalyn Yalow was awarded the Nobel Prize in Physiology or Medicine in 1977 "for the development of the RadioimmunoAssay of peptide hormones". (Solomon Berson had passed away in 1972 and Nobel prizes are not given posthumously.) She was awarded half of the monetary prize and the other half was shared by two men for their work on brain peptide production. RadioimmunoAssays are still used today to detect very small quantities of substance. The development of the RadioimmunoAssay technique opened the door for others to develop similar methods, like ELISA, to test proteins but without the use of radioactive substances. (7, 10)

What Are ELISAs and When Were They Developed?

In France, Sweden, the Netherlands, and the United States from 1966 to 1970, research on coupling antigens or antibodies with enzymes, such as alkaline phosphatase, glucose phosphatase, and glutaraldehyde, was completed and published. At the 1970 meeting of the European RadioimmunoAssay Club, it was suggested that use of these bulky protein enzymes might hinder the antigen-antibody interaction. However, in 1971, Peter Perlmann and Eva Engvall at the University of Stockholm, Sweden, published their first paper on Enzyme –Linked ImmunoSorbent Assay (ELISA) showing they could quantify the amount of IgG in rabbit serum with alkaline phosphatase as the reporter label.

The same year, Anton Schuurs and Bauke van Weemen in the Netherlands published a paper showing that with the Enzyme ImmunoAssay (EIA) method, they could quantify the amount of human chorionic gonadotropin in urine with horseradish peroxidase coupled with glutaraldehyde as the reporter label. These assays were considered highly sensitive and specific and compared favorably with radioimmunoassays but without using radioactive substances.

ELISA/EIA test kits began to be produced soon after these papers were published. For example, in 1976, Organon Teknika Company developed and marketed a highly successful EIA system for the hepatitis B

surface antigen using the microtiter plate (known as the 96 well plate). Other improvements which followed were the automated pipetting devices, multichannel pipettes, and microtiter plate readers and washers. By the 1980s, fully automated test instruments were being manufactured and are used today in medical laboratories. (6, 10)

How Is the ELISA Method Used?

The ELISA method has been used to detect hepatitis B, rabies, and HIV through antibodies in the blood serum, just to name a few diseases, and to measure amounts of various other proteins in the blood serum, such as hormones, toxins, and allergens. The method works by attaching a primary antibody or an antigen to a solid surface such as the wells of the microtiter plate, blocking unbound sites with a non-interacting protein, washing away unbound proteins, adding test sample antigen if a primary antibody was attached to the plate or adding test sample antibody if antigen was attached to the plate, washing away unbound protein, adding enzyme-conjugated secondary (detection) antibodies, washing away any unbound antibodies, developing color by adding substrates that react with the enzymes, and reading the results. (1, 11)

What Are the Types of ELISA?

Using this protocol, there are five types of ELISA methods which include a) indirect ELISA, b) sandwich ELISA, c) direct ELISA, d) competitive ELISA, and e) multiplex ELISA. The indirect (to detect antibodies in the sample) and the sandwich (to detect antigens in the sample) ELISA tests are the two most common types used. (11)

a) The **indirect ELISA** method begins with a sample of known antigen being bound to the wells of a microtiter plate. Then the other unoccupied sites in each well are bound by a concentrated solution of non-interacting protein, like casein or bovine serum albumin, to block or prevent other proteins in the test sample from adhering. The wells are then washed to remove any unbound antigen and non-interacting protein. Next the test sample of serum containing the primary antibodies is added to each well. Antibodies could be HIV, rabies, or hepatitis antibodies, for example. After a time, the plate is washed to remove any primary antibodies that did not bind to the known antigen. An enzyme-linked secondary antibody is added next to bind to the test sample antibodies. The enzymes on the secondary antibodies are proteins, such as horse radish peroxidase or alkaline phosphatase. The wells are washed to remove any unbound secondary antibodies. A substrate is then applied which is converted by the enzyme to give a color or fluorescence or electrochemical signal that can be read by a spectrophotometer, spectrofluorometer, or electrochemical device. Substrates used with peroxidase include ABTS (2, 2'-azo-bis (3-ethylbenzthiazoline-6-sulfonic acid))

which turns green, OPD (o-phenylenediamine) which turns orange, and TMB (3, 3'5, 5'-tetremethylbenzidine base) which turns blue. pNPP (pnitrophenylphosphate) substrate added to alkaline phosphatase turns yellow. The amount of color produced is proportional to the amount of primary antibody bound to the antigen proteins on the bottom of the wells.

For an excellent 2 minute animation of the indirect ELISA method and test results presented by Dr. Cary Engleberg, MD, Professor, University of Michigan Medical School, google 'Enzyme Immunoassay', then scroll down to 'Enzyme Immunoassay (EIA) to Detect Antigens – You Tube' and click on it. It will begin to play (Sandwich ELISA method) but click on the right panel (top) on 'Enzyme-Linked Immunosorbant Assay (ELISA) –Multi-Lingual Captions by openmichigan' and play the Indirect ELISA method instead.

This indirect ELISA method was the first screening test widely used for testing for HIV infection because of its high sensitivity. If a person has been exposed to the HIV virus through unprotected sex, contaminated and shared needles, or contaminated blood transfusions, the B-cells recognize a foreign antigen on the surface of the virus and produce an antibody to attach to it as part of their humoral immune response. The B-cell makes plasma cells which release the antibodies into the bloodstream and memory cells containing antibodies on the cell surface for future possible exposure to the same antigen. Normally the circulating HIV antibodies would attach to the invading HIV virus and disable them, but some of the HIV virus tends to hide inside the white blood cells away from the antibodies. Inside the cell, the virus can lie dormant and eventually replicates causing full-blown AIDS.

Using the indirect ELISA method, testing would begin by coating the well plate with inactivated HIV antigens. Non-interacting proteins would be added to bind to unoccupied sites and then washed to remove unbound antigens and non-interacting proteins. Then the patient's blood serum possibly containing the HIV antibodies would be added to the well plate. After washing away any unbound antibodies (those not associated with HIV infection), a secondary antibody, an anti-human immunoglobulin antibody coupled to an enzyme, is added to bind to the human antibodies. After washing away any unbound secondary antibody, a substrate is added which changes color when cleaved by the enzyme attached to the secondary antibody. The plate is read and a number is reported. The most controversial aspect of the HIV test is where to determine the cut-off point between positive and negative results. If the results indicate a positive result, further testing, such as by Western blot, would be done to confirm the positive diagnosis of HIV infection. (1, 2, 6, 11)

b) The **sandwich method** begins by binding a known antigen-specific antibody (called the capture antibody) to the surface of the wells of the microtiter plate. The other unoccupied sites in each well are blocked or prevented from binding to other proteins in the test sample by adding a concentrated solution of non-interacting proteins. The wells are washed to remove any unbound capture antibody and non-interacting protein. Next the test sample antigen is added and allowed to bind to the antibody. Any excess and unbound antigen is then washed away. An enzyme-linked antibody (called the detection antibody) is added which is also specific to the same antigen. The antigen to be measured must have two antigenic sites, one for the capture antibody and one for the detection antibody. Any excess and unbound detection antibody is then washed away. A substrate is added which interacts with the enzyme and is converted to a color or fluorescence or electrochemical and the results are read using the appropriate device. As with the indirect ELISA method, the amount of color produced is proportional to the amount of antigen bound to the capture and the detection antibodies.

The sandwich ELISA protocol could be used when trying to determine how much hormone, such as testosterone, estrogen, and progesterone, is present in a test sample. For instance, if one wanted to know the amount of testosterone present over the lifetime of a man or the amount of estrogen and progesterone over the monthly menstrual cycle of a woman, the sandwich ELISA method could be used. The sandwich assay is often used to test for presence of human chorionic gonadotropin hormone (hCG) in the bloodstream and urine of women and, if present, indicates pregnancy. Home pregnancy test strips use the sandwich ELISA method and can detect hCG in urine after implantation of the embryo into the uterus, which occurs six to twelve days after fertilization. The sandwich ELISA method is also used by the food industry to test for potential food allergens, by toxicologists to test for pathogenic toxins, and by physicians to test for minute amounts of protein in the blood, such as alpha fetoprotein in the mother's blood indicating possible birth defects in the fetus, like spina bifida. (1, 6, 11, 12)
For an excellent 2 minute animation of the sandwich ELISA method and test results presented by Dr. Cary Engleberg, MD, Professor, University of Michigan Medical School, google 'Enzyme Immunoassay', then scroll down to 'Enzyme Immunoassay (EIA) to Detect Antigens – You Tube' and play the Sandwich ELISA method.

c) The **direct method** is quick and avoids the possible problem of cross-reactivity of the secondary antibody with other proteins in the antigen sample. For the direct ELISA test, the wells of the microtiter plate are coated with the test sample antigen, unoccupied sites are blocked with non-interacting protein, and washed to remove unbound antigen and non-interacting proteins. Next, an enzyme-labeled antibody specific for the antigen is added. The

unbound antibody is washed away and a substrate is added to effect a change in color, fluorescence, or electrochemical interaction which can then be read by the appropriate device. The amount of color produced is proportional to the amount of antibody bound to antigens in the wells.

d) The **competitive ELISA** method is an advantage when two antibodies are not available to bind to the antigen as is necessary in the sandwich ELISA protocol and/or when only crude or impure samples are available. For the competitive ELISA method, unlabeled antibody is incubated in the presence of the sample antigen. These bound antigen-antibody complexes are then added to antigen-coated, blocked, and washed wells. The wells are then washed to remove any unbound antigen-antibody complex. The more antigens in the sample, the fewer antibodies will be able to bind to the antigen in the well, thus its name competitive. The enzyme-linked secondary antibody is added, washed, substrate is added, and resulting color read. In competitive ELISA, the higher the sample antigen concentration, the weaker the eventual color – an inverse relationship.

e) The **multiplex ELISA** method allows for simultaneous detection of multiple tests within a single well on the microtiter plate. By coating an array of different capture antibodies in each well, corresponding different antigens in the test sample can be detected in one test using an array of corresponding detection antibodies. (1, 6, 11)

<u>What Is Immunohistochemistry and How Is It Related to ELISA?</u>

While ELISA tests are routinely used to test antigen or antibody presence in patient blood serum, the direct ELISA method and the indirect ELISA method also have been applied in immunohistochemistry. The tissue being studied is embedded in paraffin and thinly sliced with a microtome onto a glass microscope slide. In order to fluorescently tag a particular cell component, the paraffin is removed, the antigens of the tissues retrieved, and a blocking non-interacting protein is added to bind all unoccupied sites on the slide. Then the slide is washed to remove any unbound non-interacting protein. In the direct immunochemistry method, next an enzyme-linked antibody is added and then washed to remove any unbound antibody. The substrate is added and the color read and recorded. In the indirect method after washing to remove any unbound non-interacting protein, an antigen-specific primary antibody is added and then washed to remove any unbound primary antibody. Next an enzyme-linked secondary antibody is added and then washed to remove any unbound secondary antibodies. Then the substrate is added and the color read and recorded. While the direct immunohistochemistry method is much quicker, the indirect immunohistochemistry method is thought to be more sensitive.

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