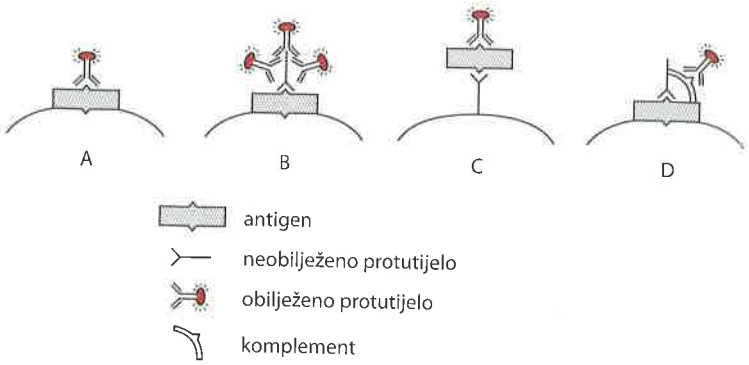
Postoji nekoliko načina izvođenja imunofluorescencije (sl. D3-15.).

U **direktnoj imunofluorescenciji** obilježena protutijela izravno reagiraju sa stanicama ili tkivima na kojima su specifični antigeni. Taj se oblik imunofluorescencije rjeđe upotrebljava, a koristan je za identifikaciju različitih mikroorganizama.

Indirektna imunofluorescencija ima dvije faze. U prvoj, s antigenima reagiraju neobilježena protutijela. Nakon što je reagiranje završeno, u drugoj se fazi dodaju obilježena antiglobulinska protutijela koja se vežu za neobilježena protutijela, dodana u prvoj fazi. Ta se metoda rabi mnogo češće, a razlozi su za to jača fluorescencija i nužnost obilježavanja samo jednoga seruma, što znatno ubrzava proces pripremanja preparata. Primjeri su indirektne imunofluorescencije dokazivanje antibakterijskih protutijela (serodijagnostika luesa, toksoplazmoze, leptospiroze) i autoantitijela (antinuklearna protutijela u bolesnika sa sistemnim eritematoznim lupusom).

Sendvič-test primjenjujemo tada kad želimo dokazati određena protutijela na stanicama (npr. receptore na limfocitima B). Suspenziju stanica ili rezove tkiva inkubiramo s otopinom odgovarajućeg antigena, a zatim dodamo obilježena protutijela protiv tog antigena. Mjesta koja fluoresciraju pokazuju nam gdje se nalaze stanice koje su membranskim protutijelima vezale antigen.

Konačno, uporabom obilježenog **antikomplementskog antiseruma** mogu se dokazati kompleksi koji se sastoje od antigena, protutijela i komplementa (v. 18. pogl.).



Slika D3-15. Imunofluorescencija. A) Direktna metoda. B) Indirektna metoda. C) Sendvič-tehnika. D) Uporaba antikomplementskih protutijela.

Na imunofluorescenciji se osnivaju i **protočni razvrstači stanica**. Načelo njihova rada potanje je opisano u odsječku D3.3.2.1.1.

D3.3.1.2.5. Obilježavanje protutijela enzimima

Protutijela se mogu obilježiti i nekim enzimima, pri čemu se najčešće rabi peroksidaza.

Umjesto fluorescentnim tvarima, protutijela se mogu obilježiti i nekim enzimima. Prisutnost protutijela obilježenih enzimima dokazuje se histokemijskim metodama, pri čemu enzimi djeluju na neku tvar (supstrat) tako da ona promijeni boju. Peroksidaza je enzim kojim se najčešće obilježavaju protutijela. Dodatkom peroksida i njegova supstrata diaminobenzidina tkivnom preparatu pojavit će se tamnosmeđa boja na onim mjestima na kojima se nalaze enzimom obilježene molekule protutijela. Prednost je enzimskih metoda u odnosu na imunofluorescenciju u tome što one ne zahtijevaju uporabu fluorescentnoga mikroskopa i što se preparati mogu čuvati mnogo dulje od onih u kojima su protutijela obilježena nekom fluorescentnom tvari.

Često upotrebljavana enzimska metoda, koja omogućuje kvantitativna istraživanja, jest **ELISA** (prema engl. *enzyme-linked immunosorbent assay*).

Postoji nekoliko načina izvođenja imunofluorescencije (sl. D3-15.).

U **direktnoj imunofluorescenciji** obilježena protutijela izravno reagiraju sa stanicama ili tkivima na kojima su specifični antigeni. Taj se oblik imunofluorescencije rjeđe upotrebljava, a koristan je za identifikaciju različitih mikroorganizama.

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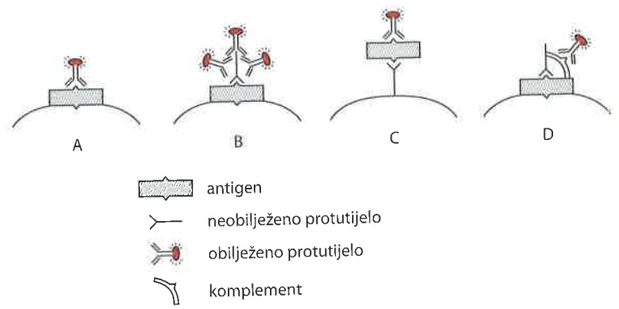

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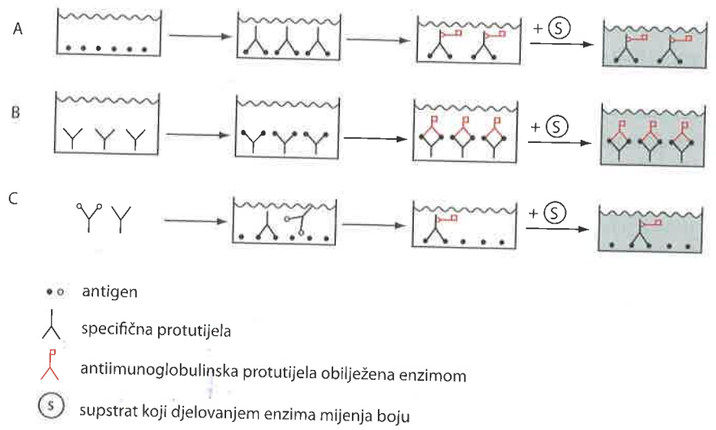
Često upotrebljavana enzimska metoda, koja omogućuje kvantitativna istraživanja, jest **ELISA** (prema engl. *enzyme-linked immunosorbent assay*).

Razrađene su različite inačice te metode: indirektni ELISA-test, sendvič-ELISA-test i kompeticijski ELISA-test.

U **indirektnom ELISA-testu** (sl. D3-16.A) antigen se adsorbira na plastičnu podlogu te se najprije inkubira sa serumom u kojemu se određuju protutijela protiv tog antigena, a zatim s antiimunoglobulinskim protutijelima obilježenima nekim enzimom. Nakon dodatka supstrata, koji pod utjecajem enzima mijenja boju, kolorimetrijskim se metodama može – na osnovi intenziteta nastale boje – odrediti količina protutijela u serumu. Indirektni ELISA-test vrlo je prikladan za dokazivanje serumskih protutijela protiv virusa HIV.

Sendvič-ELISA-test (sl. D3-16.B) prikladan je za dokazivanje izlučenih citokina, pa je dobar pokazatelj funkcije limfocita T, posebice pomagačkih. Na podlogu se najprije adsorbiraju protutijela koja prepoznaju neki epitop istraživanoga citokina, a zatim se doda uzorak koji sadržava taj citokin. Nakon ispiranja dodaju se protutijela specifična za neki drugi epitop citokina; ta su protutijela obilježena enzimom. Citokin se tako nalazi u »sendviču« između dvaju protutijela, a njegovu prisutnost pokazuje promjena boje dodanoga supstrata.

U **kompeticijskom ELISA-testu** (sl. D3-16.C) pomiješa se uzorak antigena s protutijelima, a zatim se ta mješavina prenese na podlogu na kojoj je adsorbiran antigen. Što je više antigena u uzorku, to će manje biti slobodnih protutijela koja će se moći vezati za adsorbirani antigen. Dodatkom obilježenih antiimunoglobulinskih protutijela može se odrediti količina protutijela vezanih za adsorbirani antigen.



Slika D3-16. ELISA-test. A) Indirektni ELISA-test. B) Sendvič-ELISA-test. C) Kompeticijski ELISA-test.

Metode koje se osnivaju na obilježavanju enzimima mogu se upotrijebiti i za identifikaciju antigena u nekoj mješavini. To se može postići nekom od inačica tzv. metode **Western blotting** (engl. *blot* – mrlja). Sastojci u mješavini antigena najprije se elektroforezom razdvoje, a razdvojene se komponente prenesu na nitrocelulozu opnu. Želimo li među razdvojenim sastojcima identificirati određene antigene, moramo na opnu dodati neobilježena monoklonska ili poliklonska protutijela protiv tih antigena, a potom antiglobulinska protutijela obilježena peroksidazom. Antigenske komponente, za koje su se vezala protutijela, mogu se učiniti vidljivima dodatkom supstrata, a prikazuju se poput mrlja (sl. D3-17.). Budući da je veličina mrlje razmjerna količini antigena, ta metoda može poslužiti i za kvantitativna određivanja.

DIAGNOSIS OF DISEASE BASED ON IMMUNE RESPONSE

--A VIRTUAL LABORATORY EXERCISE--

Components of the immune system called antibodies are found in the liquid portion of blood and help protect the body from harm. Antibodies can be used also outside the body in a laboratory-based assay to help diagnose disease caused by malfunctions of the immune system or by infections. This virtual laboratory will demonstrate how such a test, termed an enzyme-linked immunosorbent assay (ELISA), is carried out and some of the key experimental problems that may be encountered. Students will learn about the assay procedure and the equipment and materials that are needed. By completing this exercise, students will gain a better understanding of experimental design, key concepts in immunological reactions, and interpretation of data.

Concepts Covered

The basis of humoral immunity The foundation for ELISA Potential errors in conducting an ELISA Sensitivity and specificity of a diagnostic test

Potential Experimental Problems

ELISA is used in many laboratories to determine whether a particular antibody is present in a patient's blood sample. Although the procedure is routine and straightforward, it involves a number of variables, such as reagent selection, temperature, volume measurement, and time, which if not adjusted correctly can affect subsequent steps and the test outcome. This virtual laboratory has been developed so that when a mistake is made, you will not get the correct answer. The program keeps track of errors made throughout the experiment and generates a report at the end.

Limitations of the Test

This general test has some important limitations.
First, a positive result correctly confirming the presence of antibody does not necessarily mean the patient is sick. The body can continue to produce antibodies even though the person may have had the disease earlier and recovered.
Second, people may be poor producers of antibody or may have some interfering substance in their blood. The amount of antibody, consequently, may be too low to measure accurately or may go undetected. This result is termed a false negative.
Third, a positive result may occur if an unrelated antibody reacts with the antigen nonspecifically. Unlike a true-positive result where the specific antibody is detected, however, this positive reaction is false. Testing many patients and running tests more than once helps lab workers distinguish a true from a false result. To avoid simple experimental mistakes leading to incorrect results, scientists conduct tests using duplicate (or, sometimes, more than two) samples.

How Does This Work?

The interaction of antigen and antibody outside the body--in the laboratory--can be used to determine if a patient has an infectious or an autoimmune disease. The test measures whether a specific antibody associated with an illness can be found in a patient's blood. A positive result indicates that the antibody is there and implies that the person has encountered a particular disease.
This exercise begins with removing red and white blood cells, which can interfere with the test, from a patient's blood sample. The watery fluid that remains is called serum. A portion of serum possibly containing the antibody is allowed to react with the target antigen. A correct match causes the antigen and antibody to bind together.
Detection becomes possible when a second antibody is added. This antibody is prepared from the serum of an animal injected previously with human antibody; the human antibody in this case serves as an antigen and the animal thus produces an antibody against the human antibody. Once isolated, the second antibody can be chemically linked to a system that can produce a detectable signal.
In ELISAs, the antigen antibody complex is exposed to the second antibody, which binds to the antibody portion of the complex (against which it was formed), creating a sandwich-type structure (Figure 1). The signaling system consists of an enzyme attached to the second antibody. When the appropriate chemical is added, the enzyme converts it to a colored substance that can be measured.
This test quantifies how much enzyme is present by the amount of color produced. The more enzyme present, the more secondary antibody must be attached. The amount of secondary antibody present is determined by the amount of target, or first antibody, available. Finally, because the first antibody binds to antigen, the more antigen that is accessible, the more first antibody will be retained. The measure of color, therefore, reflects the amount of antigen initially present.

Laboratory Protocol

You are the manager of a clinical laboratory. A local physician sends you 10-ml (milliliter) vials of blood from three different patients and asks you to test each sample for supporting evidence of a chronic disease called systemic lupus erythematosus (SLE).
You must conduct an ELISA to look for antibodies characteristic of this disease. The presence of a specific antibody in a blood sample will indicate that the patient may have SLE (see Figure 1).
Use this laboratory notebook for the protocol.

Lab Notebook

Step 1
Centrifuge whole-blood samples of patients A, B, and C for 15 minutes at room temperature to get the sera.
Why?
The samples must be centrifuged to precipitate the blood cells and obtain the clear fluid known as serum. Any cells that remain will interfere with the assay and may cause a positive result to appear regardless of whether the SLE antibody is present.
An assay that lacks specificity and produces a false-positive result is not useful for making a diagnosis.

Step 2
Using the serum from patient A, prepare three dilutions as follows:
Take 1 ml of serum from patient A and add 1 ml of phosphate-buffered saline (PBS) solution. This is a 1:2 dilution.
Take 1 ml of serum from patient A and add 9 ml of PBS. This is a 1:10 dilution.
Take 0.1 ml of serum from patient A and add 9.9 ml of PBS. This is a 1:100 dilution.
Why?
Serial dilutions are made in order to determine the level of the antibody in the sample. Highly diluted samples will not appear positive if there is a low titer of antibody in the sera.
PBS is a common laboratory solution that contains table salt. The amount of salt in the buffer is about the same as that normally found in blood. The phosphate buffer keeps the solution from becoming too acidic or basic.

Step 3
Prepare an ELISA plate with 0.1 ml of the different dilutions of patient serum using an Eppendorf® pipettor.
*Note: The ELISA plate has been pretreated to bind SLE antigen to each well.
Why?
Proteins such as antigens and other biological materials can, under proper conditions, physically bind to the plastic material composing the wells of the ELISA plate.
The coating procedure must be done carefully. If too little antigen is used, bare spots will permit antibody or other protein to stick, leading to a false-positive reaction. If too much antigen is used, the excess will be able to bind SLE antibody from patient sera but then will be washed away, creating a false-negative reaction.
The addition of antigen is the crucial first step in the chain of recognition events between antigen and antibody that will end with the formation of color from the enzyme bound to the second antibody.

Step 4
Add to the ELISA plate 0.1 ml dilutions for each titer of anti-DNA primary antibody (positive control) and a buffer (negative control).
Why?
An ELISA may be subject to many errors. One is that the biological and chemical reagents used in ELISA can change with time. Another is that the ELISA is not always conducted under appropriate conditions. To rule out such problems, two controls are used. One control should always produce a positive response if the reagents and conditions are correct. The second control should never produce a positive response. If either control sample fails to react as expected, then the results for the patients' samples cannot be trusted and the assay must be repeated.
Any serum from a patient that contains the antibody for SLE will recognize the antigen in the well and bind to it. Each serum sample contains many different types of antibodies, but because they are so specific in how they react, usually no antibody will recognize the SLE antigen except the SLE antibody.

Step 5

Incubate the ELISA plate at 37° C for 15 minutes.

Why?

Incubating serum samples in antigen-coated wells helps ensure that the antibody present in the sample will interact correctly with the antigen. Because SLE is a disease of humans, the reaction usually occurs at the temperature of the human body, which is 37 degrees C. Time is important: the reaction must proceed long enough for adequate binding to occur or the measurement will be artificially low.

Problem: If the timer is set to less than 15 minutes, the proper reaction will not occur and no color will be evident at the end of the assay. The observer will record the results incorrectly as false negatives.

Problem: If the temperature is set too low, the reaction will not be completed in the allowed time. If the temperature is set too high, protein (antigen and antibody) will be adversely affected via a process known as denaturation, which diminishes its ability to interact. The results will again be recorded as false negatives.

Step 6

Remove the fluid from each well with the pipettor and wash with 0.1 ml of PBS.

Often, in a real laboratory, these washes are repeated 3-6 times prior to adding the next substance. In order to speed this example along, we have given just one rinse/wash example per step.

Why?

Washing helps remove any antibody that did not react with the SLE antigen in the well. When the fluid is removed from the well, antibody that has reacted with antigen remains attached to the well surface. Unreacted (unbound) antibody may also remain in the well in the small amount of fluid that is left behind. This unbound antibody must be removed, because the anti-human antibody added in the next step will recognize and react with any antibody remaining in the well, regardless of whether that antibody is specific for the SLE antigen. A reaction with non-SLE antibody will produce a false-positive result.

Step 7

Add 0.1 ml of buffered solution containing a secondary antibody that recognizes antibodies made in humans. Note this secondary antibody is made in a rabbit and has an attached enzyme (HRP) that will interact with the substrate in the next step.

Why?

The second antibody, unlike the first, does not recognize the SLE antigen. Instead, rabbit anti-human antibody reacts with human antibody. SLE antibody is a human antibody that may be present in a well because it is being held by antigen. The second antibody (from rabbit) will therefore recognize this antibody and bind to it. If the well has not been washed thoroughly, other human antibody may still be there and will also react with the second antibody. Reaction of a non-SLE human antibody with the second antibody will produce a false-positive result.

Step 8

Incubate the ELISA plate at 37° C for 15 minutes.

Why?

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Step 9

Remove the fluid from each well with the pipettor and wash with 0.1 ml of PBS.

Why?

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Step 10
Add 0.1 ml of buffered solution containing the chemical substrate (HRP-substrate). If there are human antibodies present, the clear substrate will turn yellow.
Why?
One of the most commonly used enzymes that can be attached to antibody is called horseradish peroxidase. The enzyme together with hydrogen peroxide acts on a chemical called ABTS (2,2'-azinobis-3-ethylbenzothiazoleine-6-sulfonic acid) to produce a yellow solution that can be estimated by eye or quantitatively measured in a spectrometer at 414 nanometers.

Step 11
Set timer for 15 minutes.
Why?
If the timer is set to less than 15 minutes, the proper reaction will not occur and no color will be evident at the end of the assay. The observer will record the results incorrectly as false negatives.