Asian Pacific Journal of Allergy and Immunology (1993) 11 : 167-175

SPECIAL ARTICLE

Diagnosis of Viruses by Immunoassays

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The search for new and better diagnostic aids over the past 10 to 20 years had led to the application of immunology to diagnostic medicine and to the creation of a new group of tests known as immunoassays. Immunoassays combine the specificity of an antigen-antibody reaction with the sensitivity of an indicator system. These are ligand assays ie binding assays that follow the law of mass action.[1] For high sensitivities these immunoassays usually make use of an antigen, hapten, or antibody labelled in some way. The most common labels are fluorescent dyes, radioisotopes, and enzymes.[1-3] The high sensitivity achieved relates to the physical and/or biochemical characteristics of the label, which releases high energy products or amplifies the signal.[1,3] Assays employing these labels may be evaluated both quantitatively and qualitatively.

A variety of arboviruses have been detected using immunofluorescence (IF).[4-8] In this immunoassay, the labels used emit luminescent light. Luminescence is categorized according to the source of energy used to excite the molecule to a high energy state which then emits light as it returns to the ground state. Fluorescence is a form of photoluminescence where photons in the ultraviolet light and visible spectrum excite molecules from a ground state to a high electron state. Upon returning to the ground state the excited molecules release energy in the form of photons of longer wavelength. Fluorophores are the molecules capable of fluorescing when stimulated into a high energy state. Examples of fluorophores are fluorescein, tetramethyl rhodamine, umbelliferone, and europium (III).[1,3] IF depends upon subjective assessment of the end result, and this technique is often laborious.[9]

Radioisotopes are by far the most commonly used labels.[10] Immunoassays using radioisotopes are usually called radioimmunoassays (RIA) when employing a labelled antigen and immunoradiometric assays (IRMA) when employing a labelled antibody.[1,11] RIAs are constructed in a heterogeneous format, measuring the amount of radioisotope labelled molecules either attached to a solid phase or precipitated as insoluble complexes during competitive inhibition assays.[1,11] RIAs have been developed and some are commercially available to detect antigens of a number of viruses, for example, herpes virus, adenovirus, vaccinia virus, measles virus, rotavirus, Norwalk virus, hepatitis virus, and several arboviruses.[11]

RIAs have great applicability and potential for automation. RIAs have provided the sensitivity to measure extremely low levels of small molecular weight compounds such as drugs and hormones.[2] RIA is more sensitive than IF.[11-14] However, IF is the superior method when considering cost, safety, and procedural difficulty, but typically the viral antigen must be tissue associated.

RIAs which rely on the emission of gamma radiation by an isotope-bound immunoreactant, have a

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number of disadvantages. For example, the fact that radioactive isotopes have an inherent rate of decay means that the radio-labelled reagents will lose their activity over time. Thus repeated relabelling, retesting, and restandardization are required. Also, RIA systems subject the users to a potential hazard. Finally, expensive equipment is needed to measure the radiation, thus restricting RIAs to central laboratories.[15]

One further requirement of RIA is a separation step. The binding of antibody to the antigen does not affect the activity of the label. In order to determine the proportion of labelled antigen bound by the antibody and the effect of unlabelled antigen upon this distribution it is necessary to separate free from bound antigen. This separation step increases the complexity of the assay and is a major step in automation of RIA.[3]

Thus we can see that neither IF or RIA meets the criteria of a good diagnostic test, ie speed, sensitivity, specificity, accuracy, safety, inexpensive reagents, potential for automation, long reagent shelf life, potential for field or office use, and broad applicability.[2] For these reasons there has been a great deal of interest in developing assay systems which would retain the advantage of RIA but avoid some of its inherent problems. The search for an assay system which can meet these criteria has led to the investigation of homogeneous and heterogeneous enzyme immunoassays (EIA). However, at the present time the method which has come closest to achieving this goal is heterogeneous EIA, also known as enzyme linked immunosorbent assay (ELISA).

The basic ELISA depends upon two assumptions: (i) that antigen and antibody can be attached to a solid phase support and yet retain immunologic activity, and (ii) that either antigen or antibody can be linked to an enzyme and the complex retains both immunological and enzymatic activity. Experience has shown that these assumptions are true for many antigen-antibody systems.[9] The first application of this conjugation in 1966 was the detection of antigens in tissues using a peroxidase labelled antibody.[16] An EIA for the detection of antibody followed shortly.[17] The next major EIA advance was the discovery that antigen or antibody could be adsorbed to synthetic solid phase substances. The solid phase permitted detection of soluble proteins using a heterogeneous format. This variation on the solid phase RIA designated ELISA was reported in 1977 by Engvall and Pearlmann[18] and by Van Weemen and Schuurs.[19]

Advantages of EIA (ELISA)

In general, EIAs are very sensitive due to the amplification characteristics of enzymatic processing of substrate.[2,3,9,16,17] The equipment of EIA is reasonably inexpensive and widely available.[1-3,9,16,20] Normal laboratory equipment (e.g washing bottles, pipettes, vacuum devices, etc) is sufficient for processing a sample using a heterogeneous procedure. A high volume diagnostic laboratory will wish to incorporate semi-automated or automated systems. Most EIA methods may be evaluated qualitatively or quantitatively. Qualitative assessment is made with the human eye, while quantitative assessment involves the use of spectrophotometric assessment. Manipulation of an EIA is simple and results are obtained rapidly and free of radiation hazards.[1-3,9,16,20] EIAs have also been shown to be cost effective under most circumstances.[21]

Disadvantages of EIA

The disadvantages of the EIA can be dichotomized into those that are unique to EIA and those that are shared by other immunoassays but are problematic for the EIA. The commonly cited disadvantages unique to EIA are: first, the measurement of enzymatic activity is indirect by virtue of the necessity for measuring the substrate degradation production. Therefore, the EIA does not provide an immediate end-point measurement that is characteristic of IF or RIAs. Second, the assay may be influenced by endogenous biological components; for example, enzyme inhibitors, endogenous enzymes, biochemicals that mimic the substrate degradation product may be present in the sample being tested or in the diluents used in the assays.[1-3,9,16,20] Issues that are problematic for all immunoassays include: (i) variation in binding efficiency of the solid phase, (ii) non-specific increases in background levels due to inappropriate immunological reactions or formation of protein complexes, and (iii) instability and variation associated with certain parameters of the assay procedure.[1-3,9,16,20] These problems reduce the accuracy of EIA.[3]

Important considerations for a successful ELISA

There are a number of variables which enter into the development of a successful ELISA system. These include reagents, enzymes, their conjugation methods, solid phase, non specific activity, and quantitation of reaction.[17]

Reagents

A basic requirement for a successful ELISA system is antibody specific for the antigen being measured. Antisera which are adequate for less sensitive tests such as complement fixation are often not specific enough for ELISA in that cross reactions will be noted with other viruses.[16] In general, antisera used in direct ELISA system must be prepared by the immunization of animals with highly purified anti-

gen.[22] Fortunately, the sensitivity of ELISA is such that once a satisfactory antiserum is prepared, it can be used at extremely high dilutions, so that the reagent can be conserved. For example, goat antisera for human rotavirus can be used at 1 : 100,000 allowing for 1,000,000 tests to be performed from 1 ml of antiserum.[23]

In the case of the indirect tests, it is preferable that one of the two antisera used be made from highly purified reagents. The second antiserum can be less specific. For example, high titred human infection serum can be utilized. It is preferable that more specific reagent be used to coat the solid phase and the infection serum be used as the second antibody.[17]

In most cases, whole serum can be used for the capture and unlabelled second antibody. Optimal dilutions are established by checkerboard titration.[24] In general, most hyperimmune sera can be utilized at dilutions of 1:4,000 to 1:100,000, while infection sera can be utilized at dilutions of 1:400 to 1:2,000. However, in some cases non-specific reactions due to the IgM fraction of the reagents[25] necessitate the use of the IgG fraction of the sera. The IgG fraction is prepared by precipitation of the serum in ammonium sulphate and, after dialysis in 0.01 M phosphate buffer, removal of the IgM fraction by passage through a positively charged ion exchange column.[26] As in the case of whole serum, the optimal concentration of the assay should be determined by checkerboard titration. In most cases this optimal concentration of IgG will be between 0.1 μg/ml and 1 μg/ml.

Enzymes

Sensitive assays can result from the use of enzyme labels due to the amplification effect of the enzyme.[3] Reagents are relatively cheap, and can have a long shelf life. Enzymes can function as labels as their catalytic properties allow the detection and quantitation of extremely small quantities of immune reactants. Consequently, multiple simultaneous assays are possible, labels may be prepared using wide variety of conjugation techniques, and a number of systems for detecting enzyme activity may be used.[3] The equipment is inexpensive, widely available, no radiation hazards are involved in the labelling or disposal of wastes, assays are readily automated, and can be extremely rapid.[3] The main disadvantage is the difficulty in measuring the enzyme activity, and the changes that may occur in the enzyme activity due to plasma constituents.[3] Ideally, an enzyme label should be soluble, stable, available cheaply in high purity, and should have high specific activity.[16] It should also be absent from biological fluids, should have an assay method that is simple, sensitive, rapid and cheap, and should be capable of retaining activity while undergoing appropriate linkage reactions.[16] Homogeneous EIA should be capable of inhibition or reactivation when antibody binds to the enzyme-hapten conjugate, and the assay conditions should be compatible with hapten-antibody binding.[16] In practice, few if any enzymes possess all these properties. The choice of enzyme employed should be dictated by the nature of the assay. For instance, in ELISA the enzyme activity is usually measured on the washed bound phase. Endogenous enzyme and factors interfering with enzyme activity may be removed by this washing procedure so the absence of these factors from serum is not an absolute requirement in this type of assay. However, homogeneous assays are more susceptible to this type of interference, so it is very desirable to use an enzyme absent from, and unaffected by factors present in biological samples.[3]

Horseradish peroxidase, beta-galactosidase, and alkaline phosphatase are the most widely used enzymes in heterogeneous systems.[27] Other enzymes used for heterogeneous systems include glucose oxidase, catalase, acetylcholinesterase, carbonic anhydrase, and glucoamylase. Lysozyme, malate dehydrogenase, glucose 6-phosphate dehydrogenase, and beta-D-galactosidase are commonly used in homogeneous assays.[3]

Conjugation methods

All enzyme immunoassays utilize an enzyme bound to either an antigen or, more commonly, an antibody molecule. Successful coupling involves the maintenance of both enzyme activity and immune activity following the conjugation reaction. Most coupling procedures utilize a bifunctional reagent such as glutaraldehyde[28] or sodium m-periodate.[17] In the case of glutaraldehyde, coupling probably occurs by the reaction of the aldehyde group with the epsilon-amino groups of the antibody and the enzyme. Formation of enzyme-protein conjugates using the one-step and two-step glutaraldehyde methods is illustrated in Fig. 1.[3] This coupling can be most efficiently achieved in a two-step method which involves first activating the enzyme with glutaraldehyde, removing the excess coupling agent by column chromatography, and then adding the immunoreactant.[26] While this method has a higher yield because of decreased self-coupling, it is often more convenient to use a one-step method, which involves the simple mixing of enzyme, immunoreactant, and coupling agent with subsequent dialysis of the reaction mixture to remove coupling agent.[16] The one-step method will yield adequate conjugates for use in the indirect ELISA system. However, for the direct system, the two-step method is often preferable procedure for conjugation.[17]

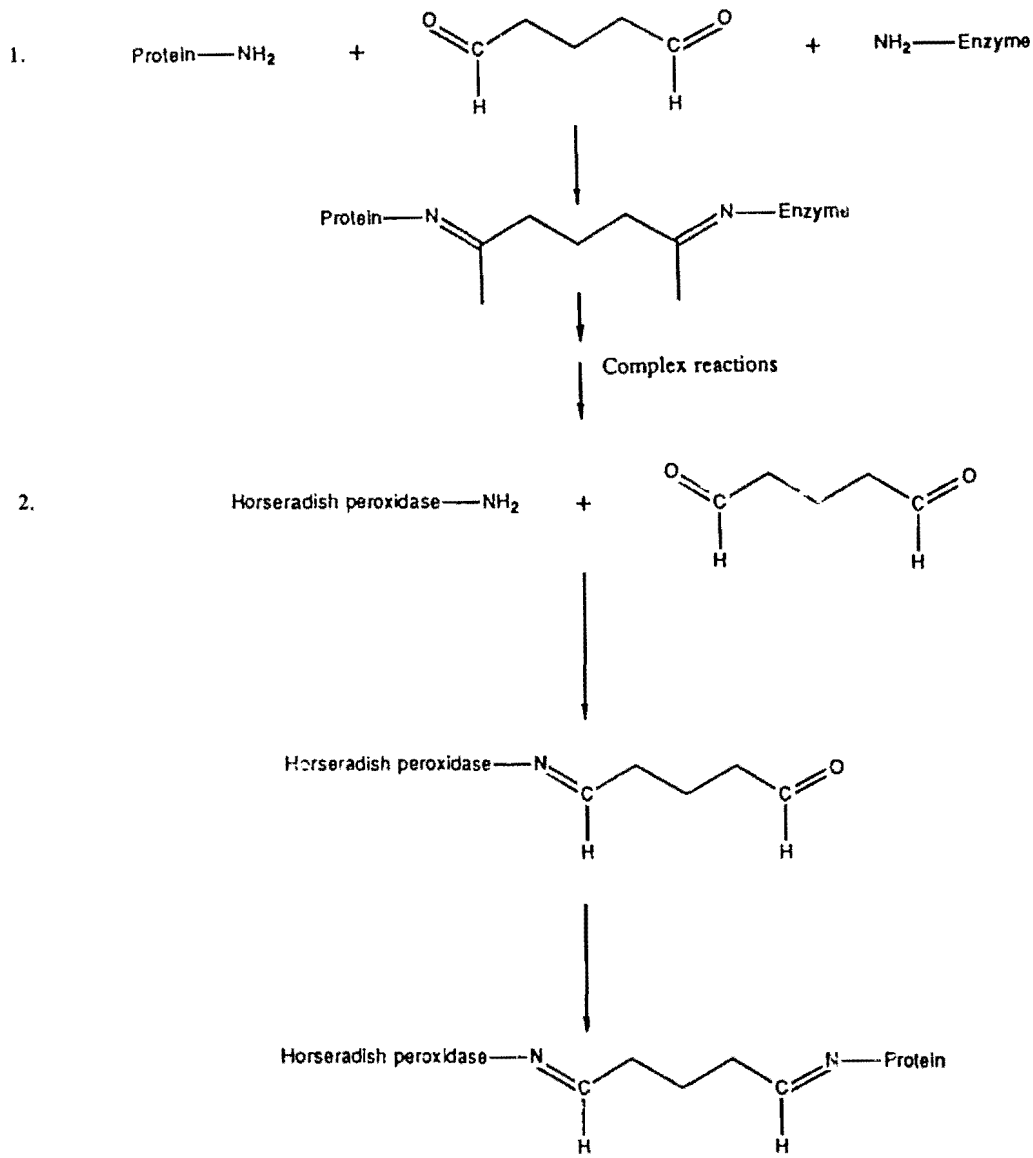

Fig. 1 One and two-step methods for the formation of enzyme-protein conjugates.

An alternate conjugation method involves the use of sodium m-periodate to conjugate the amino groups of antibody with the active aldehyde groups of the polysaccharide portion of the enzyme. Formation of enzyme-protein conjugates using the periodate oxidation method is illustrated in Fig. 2.[3] This method has the advantage of allowing for controlled conjugation, thus offering optimal ratios of antibody to enzyme.[17] However, this process is limited to use for enzymes, such as peroxidase, which contain significant amounts of glycoprotein. Utilizing this enzyme, highly efficient conjugates have been made with immunoglobulins from a number of animal species. In addition, peroxidase is inexpensive and widely available.[17]

In addition to the above methods, other agents such as 4 *N, N'*-o-phenylenedimaleimide and m-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) have been shown to be efficient coupling agents.[17,29] Formation of enzyme-protein conjugates using the maleimide method and MBS is shown in Fig. 3 and Fig. 4, respectively.[3] However, conjugates made from these agents have

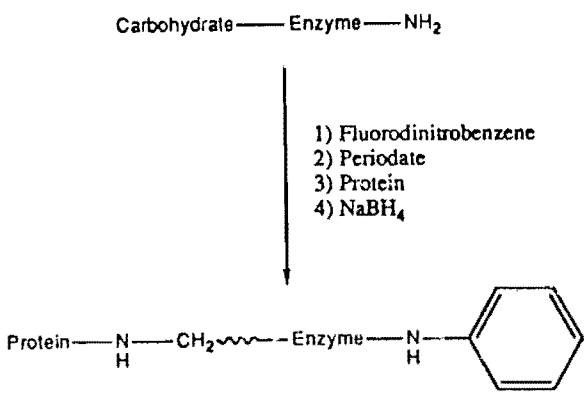

Fig. 2 Periodate oxidation method showing enzyme-protein conjugates

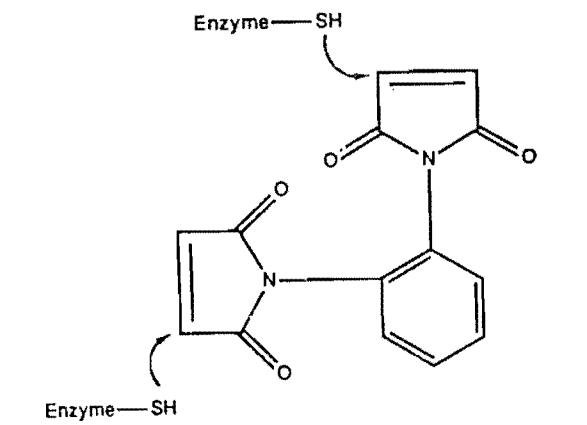

Fig. 3 Maleimide method showing enzyme-protein conjugates

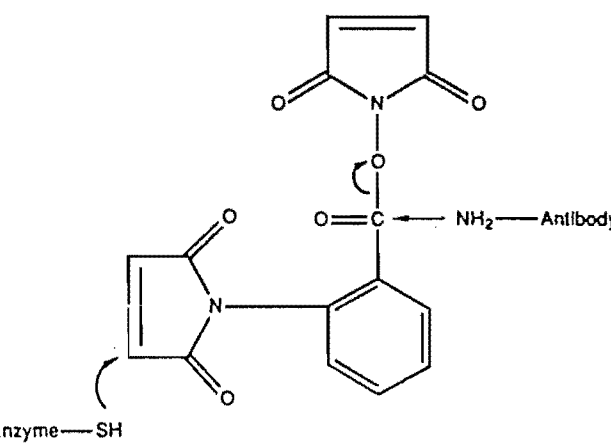

Fig. 4 MBS method showing enzyme-protein conjugates

not yet received widespread use in ELISA systems and their efficiency in these systems remains to be determined.

Solid phase

The solid phase employed in the ELISA is the most critical but least understood component in determining variation and accuracy of an assay. The development of solid phase supports has greatly expanded the versatility of ELISA. Although polystyrene microtitre plates[30,31] and tubes[32] have been the most commonly employed solid phase to which antigen or antibody is passively adsorbed, other supports such as polystyrene beads or cuvettes[2] have also been described. A number of authors have described the covalent coupling of antigen or antibody to the solid phase. The solid phase supports for covalent binding have included cellulose, isothiocyanate discs[33] and polyacrylamide.[2,31] The efficiency of binding a protein to the plastics varies with the source of the plastic and the method of adsorbing the protein to the solid phase. Both chemical and hydrophobic techniques are used to attach proteins to the solid phase.[1-3,9,16,34] Depending on the type of protein, hydrophobic adsorption will result in 3% to 20% of the protein from a 0.5 microgramme to 2.0 microgramme per ml protein solution attaching to polystyrene, approximately 80 to 100 ng of protein per 1.7 cm^2 of polystyrene.[35] Subsequent washing may result in desorbing up to 60% of the bound protein hydrophobically attached to plastic surfaces.[32,33,36-39] Chemical fixation of protein to plastic yields greater adsorption and eliminates desorbing.[32,35,36] Variation in binding efficiency occurs in commercial lots of plates.[2,20,37,40] Microtitre plates may differ in thermal conductivity characteristics which can result in systematic errors (bias).[37,38] These thermal changes

contribute to the "edge effect" of microtitre plates reported in some studies.[37,38] For problems concerning the solid phase, the general recommendations are not to use the wells of the perimetre of the microtitre plates, pre-test several commercial sources and individual lots of microtitre plates, and consider chemical fixation.

Non-specific reactions

Inhibition of non-specific binding of protein or undesirable immunological reactions is a necessary consideration when designing an EIA.[20] Non-specific ELISA reactions can occur by a variety of mechanisms. Biological fluids or tissue homogenates may contain macromolecules that are capable of aggregating. Such aggregates can complex sterically or electrostatically with the solid phase macromolecules.[2,3,16,20] The specimen or the diluent for the assay may contain immunoglobulins that react with common antigenic sites found on "unrelated" proteins. Non-ionic detergents, at concentrations of 0.05% to 2.0%, are used in assay diluents to eliminate hydrophobic attachment of proteins to the solid phase.[2,3,16,20] The commonly used detergents are polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene octylphenol (Triton X-100), and octyl phenol ethylene oxide (Nonidet P-40).[2,3,9,20,41]

Non-specific protein-protein complex formation is a major cause of false positive results. Complex formation can be reduced or eliminated by adding at least one of the followings to the assay diluents: (i) proteins with a low pI (e g bovine albumin or gelatin), (ii) mild reducing agents (e g N-acetyl cysteine), and (iii) anion polysaccharides (e g dextran sulphate).[20,33,34,42]

Because of these non-specific reactions, it is advisable to confirm each positive reaction with a second test. This can be accomplished by means of a blocking test in which the antigen is incubated with immune serum from a source independent of the immune reactants used in other parts of the assay. Such serum should reduce activity due to the antigen while a non-immune serum should not reduce specific activity.[24] A simpler confirmatory test consists of testing each specimen in wells coated with non-immune serum from the same animal that was used to prepare the capture antibody. A specimen containing specific antigen activity will yield color only in the well coated with the capture antibody, while a specimen containing antiglobulin activity will react in the well coated with the non-immune serum in addition to the well coated with the specific antiserum.[17]

Further reduction in the final amount of non-specific reactions can be effected by altering the incubation times and temperatures of various procedural steps of the assay.[20,43] Altering the kinetics of the assay to improve the specificity is based on the concept that, at a given temperature, immunological reactions of high avidity occur faster than non-specific reactions. Unfortunately, using this tactic to improve the specificity can be accompanied by a reduction in sensitivity.

Quantitation of reaction

The rate of an enzymatic reaction is dependent on a number of factors including substrate concentration, temperature, and pH.[21] Since the variables can be difficult to control under laboratory conditions, it is necessary to include in every quantitative ELISA a number of control specimens with known quantities of antigen. When a curve is constructed from the optical density values of these control specimens, accurate quantitation of unknowns can be performed.[44] In the case of qualitative assays in which the goal is simply to determine the presence or absence of an antigen in a specimen, uniform sensitivity is ensured when weakly positive controls with pre-determined amounts of antigen are added and when the positivity of a specimen is determined by comparison to these controls.[17]

Formulation of ELISA system

1. Competitive EIA for antigen (hapten)

Labelled antigen competes with unlabelled antigen for binding to a limited quantity of antibody. The antibody bound-antigen is separated from the free antigen by the use of solid phase antibody or a second antibody with specificity for the first. The enzyme activity in either the bound or free fraction is determined and related to concentration of the unlabelled antigen.[45] The procedure is analogous to the classical RIA method.[46] In the sequential saturation variant of the competitive assay, the addition of the labelled antigen is delayed until the binding between the antibody and the unlabelled antigen is complete.[47] This method is analogous to the sequential RIA.[2,48]

2. "Immunoenzymometric" assay for antigen

Antigen reacts with the excess labelled antibody and, after incubation, excess solid phase-antigen is added. The solid-phase antigen reacts with the free labelled antibody remaining and, after separation of the solid phase, the enzyme activity associated with the soluble antigen is measured and related to the concentration of antigen.[49] This assay is analogous to the immunoradiometric assay.[50]

3. "Two site immunoenzymometric assay"

Solid phase antibody is incubated with the antigen to be measured and washed, and enzyme-labelled antibody is added. Enzyme

activity bound to the solid phase is proportional to the concentration of the antigen present. This method can be used only for antigens able to bind at least two antibodies.[17,51]

4. Double antibody sandwich EIA for measuring antigen

This is a variation of method 3. It involves the use of a third antibody.[52] This antibody carries the label and reacts with unlabelled second antibody already bound to the antigen. As before, the amount of antigen is found by measuring the amount of bound label.[2,16]

5. Sandwich assay for antibody detection

This method employs direct and indirect approaches. In the direct method, the solid phase antigen is incubated with a sample containing the antibody to be detected. The solid phase is then washed, and enzyme labelled second antibody is added. The second antibody is raised against immunoglobulins of the animal species in which the first antibody was raised. The amount of enzyme activity bound to the solid phase is proportional to the amount of antigen-specific antibody present.[49,53] The indirect approach uses a capture antibody in the first step.[49]

Applications of EIA

In principle, EIA can be applied to all antigen (hapten)-antibody systems. EIAs have been developed for serum proteins, drugs, hormones, and a wide variety of other antigens and antibodies directed against them.[17,54-56] More recent methods for antigens, haptens, and antibodies by heterogeneous systems have now been described.[3]

Sensitivity and specificity of EIAs

Comparison between the sensitivities of different assays are difficult to make. The sensitivity of an assay is affected by the nature of the antiserum, the assay design, and the definition of sensitivity used. The sensitivity of several EIAs has been estimated using the diagnoses of a standard bioassay as reference. The sensitivity of the EIA is expressed as a proportion of the positive reference results in agreement. For murine leukemia virus and avian myeloblastosis virus, the sensitivity of the EIA diagnosis of viremic animals is 100% of cell culture diagnosis.[57,58] For cytomegalovirus and influenza viruses, the sensitivities of the EIAs when testing clinical specimens were less than 60% of the cell culture bioassays.[20,59] Coxsackie B and adenovirus antigen detecting EIAs are capable of diagnosing 60-62% of the clinical specimens found to contain virus by cultivation.[20,54,60] A respiratory syncytial virus antigen detecting EIA is reported to have a sensitivity between 78-82% of cell culture bioassays.[20,61,62] The sensitivity of rotavirus EIA is reported to be 86-98% of the immune-electron microscopy sensitivity.[63]

Specificity is a measure of the ability of EIA to correctly identify samples that do not contain the viral antigen(s) of interest.[2,3,9,16,20] Ideally, these negative samples should contain either no viral antigens, viral antigens from a totally unrelated virus, or viral antigens from a related virus but not the virus strain of interest. By definition, viruses that are serologically related share immunological determinants.[64] Thus there is a difficulty in defining the specificity of any sensitive immunological test using polyclonal antibody. Specificities of EIAs are reported to be 100% when the negative controls are assay diluents or specimens from uninfected normal individuals.[20,54] Negative controls from individuals infected with unrelated microorganisms or non-normal controls for other reasons can cause false positive reactions.[20] Assays designed to detect one variant of a virus (eg influenza HINI) may have reduced specificity when testing samples containing related viruses.[54] Incorporating monoclonal antibodies in the antigen detecting EIA should reduce cross reactions that occur with related viruses.[20] Specificity, akin to sensitivity, is best determined first under controlled laboratory conditions. The amount of cross reactivity between related and unrelated viruses is a factor in the false positive potential of the assay. After determination of cross reactions in laboratory experiments, the specificity must be defined using field or clinical specimens. The importance of serological cross reactions is reduced if the probability of the sample containing related viruses is minimized due to epidemiological considerations such as geographical or temporal distribution of the viruses.[59] The EIAs constructed to detect Coxsackie B virus, adenovirus, and influenza A virus are specific; they do not produce false positive results when testing specimens containing unrelated viruses.[20,54,55] However, all types of EIAs do demonstrate cross reactivity with various related virus strains.

To summarize, EIAs are extremely important in the diagnosis of viral infections. They are likely to be used in future as qualitative, semi-quantitative, and quantitative assays for screening antigens derived from microorganisms and antibodies directed towards them. They may be used as an alternate to RIA methods using tritium labels, and as labels for use in immunohistochemistry.They will also be useful in laboratories lacking facilities for counting radioisotopes, and in countries where the use of radiolabels is politically undesirable. They may also be used for homogeneous assays of low molecular weight compounds present in biological samples at a relatively high concentration. However, further research should be

directed for understanding the influence of the degree of labelling, the site of cross linking, and the nature of the bridge on the performance of enzyme labels. More studies are needed for strict comparisons of the merits of different enzymes, substrates, and detection systems, and for understanding the effects of biological samples on the activity of enzyme labels.

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