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Enzyme-Linked Immunosorbent Assay (ELISA): A Practical Tool for Rapid Diagnosis of Viruses and Other Infectious Agents

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Tissue culture techniques are inadequate to diagnose some viral infections. Thus, solid-phase immunoassays have been developed for the direct detection of viral antigens in clinical specimens. While radioimmunoassays (RIA) have attained widespread use, solid-phase enzyme-linked immunosorbent assays (ELISA) offer a number of advantages over RIA systems. ELISAs can be established with approximately the same sensitivity as radioimmunoassays without utilizing unstable, gamma-emitting isotopes.

However, before ELISA systems can obtain widespread usage, a number of aspects of the test must be optimized. These include the preparation and use of reagents, the nature of the solid phase, the choice of enzyme, and the enzyme-antibody conjugation method. With the solving of these problems, ELISA should attain widespread usage for rapid diagnosis of a large number of infectious agents.

In the past, infectious diseases have been diagnosed by the cultivation of an infecting agent from a body site. The development of efficient cultivation techniques has allowed reliable detection of a large number of bacterial, viral, and fungal agents. However, for some infectious diseases, standard cultivation techniques have not yielded an etiologic agent. For example, while it had been established by epidemiological means that hepatitis can be transmitted by a number of routes, tissue culture cultivation of specimens which were known to be infectious failed to yield a causative agent [1,2,3]. Similarly, while it had been shown that gastroenteritis in infants and young children is caused by a non-bacterial infectious agent, extensive attempts at cultivating such agents have proven fruitless [4,5]. In addition, cultivation techniques generally require the lapse of a substantial period of time before a diagnosis can be made. In the case of many viral and fungal agents, diagnosis based on the cultivation of an agent often cannot be made with sufficient rapidity to be of use in the management of an acute illness.

Because of these shortcomings, there has been a great deal of interest in developing means of detecting infectious agents without relying on cultivation. One method of detecting viral agents is immune electron microscopy [6]. Two agents which cause

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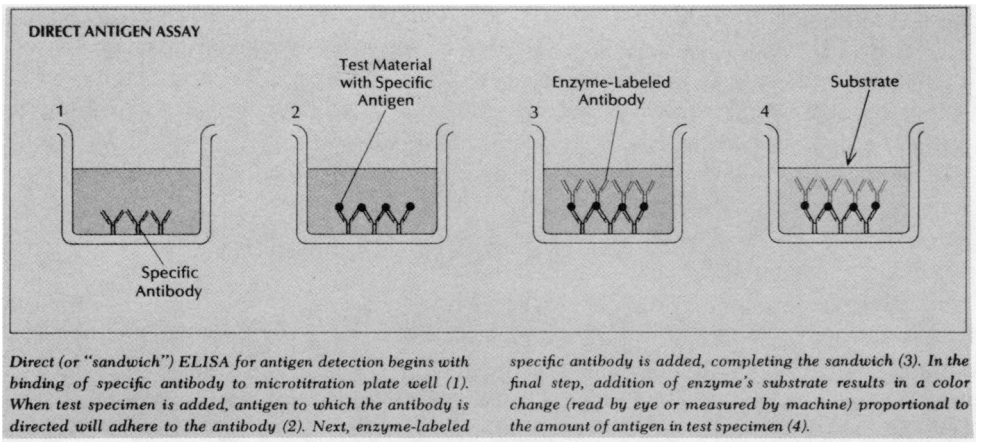
hepatitis in humans, hepatitis A and B [3,7], and two agents which cause diarrhea, rotavirus and Norwalk virus [9,10], were first investigated using electron microscopic techniques, and electron microscopy was used to elucidate the basic epidemiology of these agents. However, while electron microscopy represents an important advance in the understanding of viral infections, the need for sophisticated technology and interpretation has limited the general usefulness of this technique. Thus there has been great interest in other means of detecting and identifying non-cultivable antigens. While a number of such techniques have been developed, the one that has attained the most widespread usage for detection of hepatitis A and B antigens has been radioimmunoassay (RIA). The reasons for the success of RIA include the sensitivity and objectivity of the assay and the fact that a large number of specimens can be tested at one time. However, radioimmunoassays, which rely on the emission of gamma-radiation by an isotope-bound immunoreactant, have a 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 is required. Also, RIA systems subject the users to a potential radiation hazard. Finally, expensive equipment is needed to measure the radiation, thus restricting RIAs to central laboratories [10].

For these reasons there has been a great deal of interest in developing assay systems which would retain the advantages of RIA but avoid some of its inherent problems. The method which has come closest to achieving this goal has been the technique of enzyme immunoassay, also known as enzyme-linked immunosorbent assay (ELISA). ELISA is similar to RIA except that an enzyme is used as the immunoglobulin marker in place of a radioactive isotope. Since a single molecule of enzyme can react with a large number of substrate molecules, a very small amount of enzyme-labelled immunoglobulin can react with substrate to provide a visibly colored reaction. Thus expensive detection equipment is not needed. Also, enzyme-antibody conjugates can be prepared which have negligible loss of activity after long periods of storage, thus obviating the need for constant relabelling and retesting of reagents [9–12].

ELISA systems can be devised to measure either antigen or antibody by means of a number of system designs. In the case of the detection of an infectious agent, it is generally more convenient to label the antibody than the antigen, since the latter might not be available in purified form [36]. Thus a binding or sandwich assay is commonly used for the detection of antigen in bodily fluids. The simplest such system is a direct or one-antibody sandwich, shown in Fig. 1. In this system, which is analogous to the RIA widely used for the detection of hepatitis B antigen [13], an antibody, referred to as the capture antibody, is used to coat a solid phase. After a suitable incubation period, the unbound antibody is washed off and the test specimen is added. After another incubation period, the excess specimen is washed off and an aliquot of enzyme-labelled antibody is added. This will bind to any antigen bound to the capture antibody on the solid phase. After washing off of the unbound enzyme-labelled antibody, substrate is added. Enzyme bound to the solid phase by the previous steps will convert the substrate to a visible form which can be seen by the naked eye or measured in a spectrophotometer. This system has the advantage of requiring only a single antibody. Direct, single antibody ELISA systems have been devised for the detection of hepatitis A [14], hepatitis B (surface antigen and "e" antigen) [15,16], and human rotavirus [17].

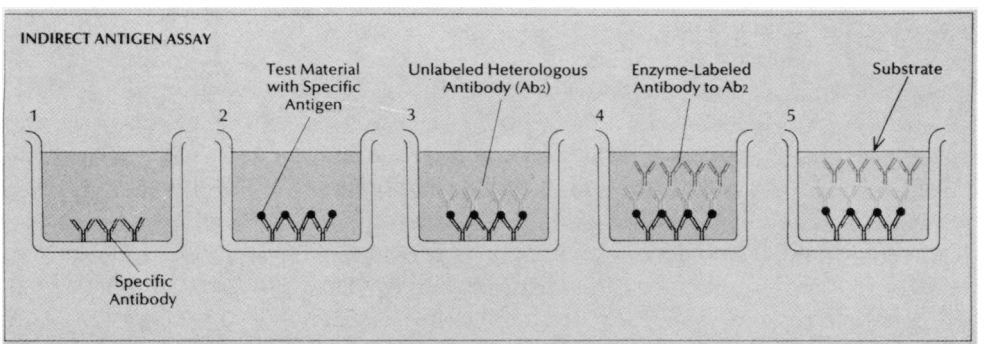
One disadvantage of this single antibody approach is that it requires the laboratory to have an enzyme-labelled reagent for each antigen to be tested. It would be more convenient to require only single labelled reagent, since this could be made commer-



Direct (or "sandwich") ELISA for antigen detection begins with binding of specific antibody to microtitration plate well (1). When test specimen is added, antigen to which the antibody is directed will adhere to the antibody (2). Next, enzyme-labeled specific antibody is added, completing the sandwich (3). In the final step, addition of enzyme's substrate results in a color change (read by eye or measured by machine) proportional to the amount of antigen in test specimen (4).

FIG. 1. Direct Antigen Assay. (*Drawing by Nancy Lou Gahan. Reprinted courtesy of* Hospital Practice, *HP Publishing Co. Inc.*)

cially available. This can be accomplished by the use of an indirect ELISA, as outlined in Fig. 2. In this case, an unlabelled, second antibody is utilized and is quantitated by the use of an enzyme-labelled anti-immunoglobulin. This method is thus analogous to indirect immunofluorescence [18]. As in that method, a single labelled antiglobulin can be used in any test system as long as the second antibody is of the same animal species. For example, we have found enzyme-labelled anti-guinea pig IgG to be an excellent reagent in any ELISA in which guinea pig antibody is used as the second antibody [19–22]. In addition, the indirect ELISA often displays increased sensitivity over the analogous direct system. Since a single molecule of unlabelled antibody is able to react with a number of anti-globulin molecules, there is an increase in the number of enzyme molecules bound per molecule of antigen with a resulting increase in sensitivity [36]. Thus we have been able to develop an ELISA system capable of measuring 1 pg of cholera toxin [22]. However, the indirect ELISA system does have an inherent disadvantage, namely that reagents prepared in two different animal species must be used to prevent the non-specific binding of the anti-



Unlike direct ELISA, indirect ELISA for antigen detection uses enzyme-labeled antiglobulin and unlabeled specific antibody from two different animal sources. Thus, after antibody from one animal species is bound to well (1) and the test specimen is added (2), unlabeled antibody from another animal species is introduced (3). Addition of enzyme-labeled antibody directed at second animal species' globulin (4) sets the stage for a color change when enzyme's substrate is introduced (5).

FIG. 2. Indirect Antigen Assay. (*Drawing by Nancy Lou Gahan. Reprinted courtesy of* Hospital Practice, *HP Publishing Co. Inc.*)

immunoglobulin to the solid phase. Thus indirect ELISA systems cannot be established for antigens such as hepatitis A, where only reagents obtained from humans and other primates are available [14].

There are a number of variables which enter into the development of a successful ELISA system. These include reagents, solid-phase enzyme conjugation methods, and non-specific activity. All of these factors must be optimized before ELISA technology can attain widespread usage.

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 [12]. In general, antisera used in direct ELISA systems must be prepared by the immunization of animals with highly purified antigen [36]. 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, our 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 [20].

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 titered human infection serum can be utilized. In our experience, it is preferable that the more specific reagent be used to coat the solid phase and the infection serum be used as the second antibody (R. Yolken et al., unpublished studies).

In most cases, whole serum can be used for the capture and unlabelled second antibody. Optimal dilutions are established by checkerboard titration [11]. In general, most hyperimmune sera can be utilized at dilutions of 1:4,000 to 1:100,000, while infection sera can be used at dilutions of 1:400 to 1:2,000. However, in some cases non-specific reactions due to the IgM fraction of the reagents [37] necessitate the use of the IgG fractions of the sera. The IgG fraction is prepared by precipitation of the serum in ammonium sulfate and, after dialysis in 0.01 M phosphate buffer, removal of the IgM fraction by passage through a positively charged ion exchange column [23]. As in the case of whole serum, the optimal concentration for the assay should be determined by checkerboard titration. In most cases this optimal concentration of IgG will be between 0.1 ug/ml and 1 ug/ml.

ENZYME

The basic requirements for a suitable enzyme for ELISA systems are that the enzyme be active when conjugated to an antibody molecule and that the conjugate be stable during storage. Two enzymes which have attained widespread usage are peroxidase and alkaline phosphatase [24–25]. Peroxidase has the advantage of being relatively inexpensive and having a number of substrate systems which produce color easily visualized by the naked eye. However, most of the proton receptors utilized precipitate after reaction, thus decreasing the sensitivity of the assay [38]. An additional disadvantage of peroxidase is that it is inactivated by bacteriostatic agents such as sodium azide, thus shortening its effective shelf life. Alkaline phosphatase, on the other hand, is stable in sodium azide and has a number of naturally occurring substrates. However, alkaline phosphatase of specific activity high enough to be used must be obtained from mammalian tissue such as calf intestine. This source is expensive and the supply is often limited. Other enzymes which have been used

include glucose oxidase, β galactosidase, and penicillinase [26,27,38]. All are widely available and have a number of usable substrates. However, their utility in ELISA systems remains to be determined.

SOLID PHASE

All immunoassays which utilize labelled reactants require a separation step in which labelled reactants not bound in the antigen-antibody reactions are removed from the reaction milieu [12]. If unreacted, labelled reactants are not separated from those involved in the specific reaction, non-specific activity will result. While differential precipitation and centrifugation have been used to achieve this separation [28], most of these methods are not practical for wide-scale usage. On the other hand, effective separation can be achieved by binding the reactants to a solid phase and washing off the unreacted labelled material [36]. A number of solid phases have been utilized, including test tubes, beads, filter papers, discs, resins, and microtitration plates of varying makeup [11,12,27,29,30,31]. While there are theoretical advantages in establishing a covalent bond between the capture antibody and the solid phase by means of a bifunctional reagent such as cyanogen bromide or glutaraldehyde, the fact that immunoglobulin molecules will bind to a number of plastics by means of hydrogen bonds greatly simplifies the process of attaching antibody to the solid phase. In most cases, simply placing a solution of serum or immunoglobulin diluted to 1–10 ug/ml in an appropriate buffer (such as 0.06 M carbonate pH 9.6) will result in a large percentage of binding over a four-hour period at 37°C or overnight at 4°C [36]. Non-specific adherence of the other immunoreactants is minimized by their dilution in a nonionic detergent such as Tween 20 and an excess of a non-immunogenic protein such as fetal calf serum or gelatin. In addition, background reactivity due to non-specific adherence is minimized by careful aspiration and washing with an excess of detergent-containing fluid [11,32]. In our hands, we have found that flexible 96 well, polyvinyl microtiter plates provide excellent binding of the capture antibody without significant binding of the other reactants. Aspiration of well contents is easily accomplished by means of a manifold which is capable of aspirating all 96 wells at once and washing by means of a similar manifold device [32]. Addition of reagents is accomplished by any of a number of multi-channel pipetting devices. The resulting color reaction can be read by the naked eye or by means of a spectrophotometer. Photometric determinations are expedited by use of a colorimeter capable of determining optical density directly in the wells of the microtiter plate [33]. The only problem encountered with these plates has been higher readings in the wells on the outer rows of the plates and an occasional erratic reading in a single well anywhere on the plate. These problems can be overcome by the avoidance of the outer rows and the running of specimens in duplicate. Even with these restrictions, 30 specimens can be run on one microtiter plate and, by using semi-automated washing and reading equipment, up to 6 plates can be run in a single day by one worker [32]. While microtiter plates of hard plastic can be used, we have found that they tend to bind less of the capture antibody, necessitating application of this antibody at a higher concentration.

One disadvantage in the use of microtiter plates is that there is a relatively small surface area available for binding. This necessitates relatively long incubation times, especially for the addition of the unknown antigen. For example, overnight incubation is required for maximal sensitivity in the rotavirus ELISA assay. It would thus be advantageous to utilize a solid phase with greater surface area when more rapid test results are required. This might be accomplished by the use of filter papers or

other porous solid-phase systems [29]. These solid-phase systems are currently under investigation in our laboratory.

NON-SPECIFIC REACTIONS

Non-specific ELISA reactions can occur by a variety of mechanisms. Non-specific binding of immunoreactants to a solid phase, which has been discussed above, can be minimized by proper choice of the solid-phase material and careful washing technique. When antibodies are prepared using crude antigen, the resulting ELISA assay might yield positive results when antigens other than the target antigen are present in the test specimen. This problem is alleviated by absorbing the antibody with the cross-reacting antigen or, preferably, purifying the antibody by affinity chromatography [38]. The use of the newly developing technology of monoclonal antibodies would also eliminate this problem. Finally, some specimens can yield false positive results due to non-specific antiglobulin activity directed against the other immunoreactants. This non-specific activity can be eliminated or markedly reduced by the addition of normal animal serum to the reaction mixture [19]. Also, since the antiglobulin is often a rheumatoid factor of the IgM subclass, its activity can be reduced by the addition of a mild reducing agent such as 20 percent N-acetyl-cysteine [37].

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 immunoreactants 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 [17]. 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.

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 [24–25] or sodium *m*-periodate [34]. 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. 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 [24]. 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 excess coupling agent [12]. Our experience indicates that this 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 the preferable procedure for conjugation.

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. This method has the advantage of allowing for controlled conjugation, thus offering optimal ratios of antibody to enzyme [34]. 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 immunoglobulin from a number of animal species. In addition, peroxidase is inexpensive and widely available. However, substrates for peroxidase are limited and have the disadvantages described above.

In addition to the above methods, other agents such as *p*-benzoquinone and N, n'-o-phenylene dimaleimide have been shown to be efficient coupling agents [27,35,39]. However, conjugates made from these agents have not yet received widespread use in ELISA systems and their efficiency in these systems remains to be determined.

QUANTITATION OF REACTION

The rate of an enzymatic reaction is dependent on a number of factors including substrate concentration, temperature, and pH [36]. Since the variables can be difficult to control under laboratory conditions, it is necessary to include in every quantitative ELISA test 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 [40]. In the case of qualitative assays in which the goal is simply to determine the presence or absence of an antigen in a specimen antigen, uniform sensitivity is ensured when weakly positive controls with predetermined amounts of antigen are added and when the positivity of a specimen is determined by comparison to these controls.

CONCLUSION

When the above variables are controlled, ELISA systems of extreme sensitivity and reliability can be constructed. The main limitation to the widespread utilization of ELISA assays is the production and distribution of the suitable reagents. The availability of such reagents, especially in the form of pre-packaged kits, will allow the clinician to rapidly diagnose a wide range of bacterial, fungal, and viral infections. This will not only allow the rational management of infectious disease but also increase understanding of a large number of disease processes. It is hoped that, in the coming years, the full potential of this simple yet powerful technique will be utilized.

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