Enzyme Immunoassays for the Detection of Microbial Antigens and Prospects for Improved Assays

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The rapid diagnosis of viral infections is an important tool in the management of patients with infectious diseases. Solid-phase enzyme immunoassays have proved to be useful tools for the direct detection of the antigens of some viruses directly in clinical specimens. Such assays have been particularly useful in the diagnosis of viral infections in the gastrointestinal and respiratory tracts. However, standard solid-phase enzyme immunoassays often do not display sufficient sensitivity for the diagnosis of all cases of viral infections. Techniques which might be utilized to increase the sensitivity of solid-phase immunoassays include the use of monoclonal antibodies to maximize the efficiency of the antigen-antibody interactions and the use of high-turnover enzymes to increase the amount of signal generated by the ensuing enzyme-substrate reactions. In addition, techniques making use of nucleic acid hybridization have a great deal of potential for the accurate detection of viral nucleic acids in human body fluids. The successful application of these techniques to the diagnosis of viral infections could lead to a marked improvement in the care of patients with suspected infectious diseases as well as to a decrease in the transmission of viral infections to high-risk individuals.

During the past years, great strides have been made in the development of practical assay systems for the rapid diagnosis of viral infections. Many of these advances have resulted from the widespread application of solid-phase enzyme immunoassay techniques to the direct detection of viral antigens in clinical samples [1–5] (Figs. 1, 2) and in the development of assay systems suitable for the detection of a wide range of viral infections of the respiratory, gastrointestinal, and genital tract [6–13]. However, while there has been significant progress in the development of solid-phase immunoassays for viral immunodiagnostics, there are limitations to the sensitivity and practicality of immunoassay systems utilizing traditional reaction formats [1,14–16]. These limitations are based on constraints inherent in the kinetics of antigen-antibody interactions. Such constraints are particularly problematic when the antibody is linked to large enzymatic molecules and the diffusion and antigen-binding capabilities of the immunoglobulin are further limited [21]. These limitations can preclude the effective use of traditional solid-phase immunoassay techniques for the diagnosis of viral infections in situations in which low concentrations of antigen are present. Such situations are likely to occur very early in the course of infection, when little viral antigen has been produced, or late in the course of infection, when much of the viral antigen is complexed with endogenous antibody and is thus inaccessable to labeled antibodies
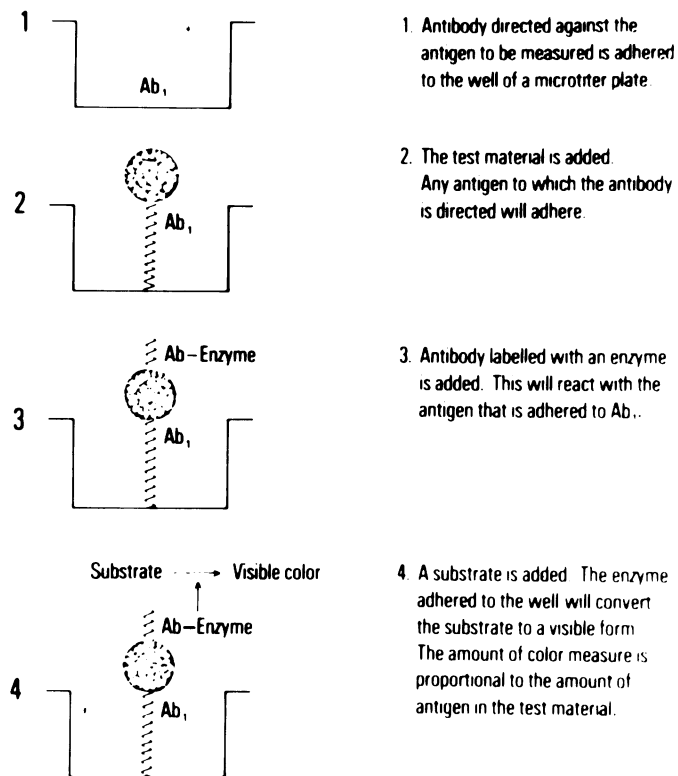
FIG. 1. Direct ELISA for antigen measurement.

[17–19]. Also, viral antigens which are sequestered in intracellular sites have little chance of reacting with labeled immunoglobulin macromolecules [20,21]. In addition to limitations of sensitivity, traditional solid-phase immunoassays require multiple incubation and washing steps to attain adequate degrees of sensitivity. The need for these reaction steps limits the use of traditional immunoassays to laboratories capable of performing the assay procedures and renders the assays difficult to perform in outpatient settings such as doctors' offices and clinics.

Recently, however, there have been a number of advances in the area of diagnostic technology which can be applied to the development of practical assays for the rapid diagnosis of viral infections. Such advances include the development of monoclonal antibodies [22,23], improved methods for the linkage of low molecular weight ligands to immunoglobulin molecules [24,25], sensitive methods for the measurement of specific anti-viral antibodies and endogenous immune complexes which develop early in the course of infection [26,27], and the application of nucleic acid hybridization techniques for microbial detection [28,29]. These advances might provide for means by which immunoassay systems can attain increased levels of sensitivity and specificity.

In the case of monoclonal antibodies, it is well recognized that the production of monoclonal antibodies with the desired specificities offers a number of potential advantages for the detection of defined viral antigens. These advantages include the fact that hybridoma technology provides for an unlimited supply of a consistent reagent which can unequivocally distinguish viral from host antigens [22,23]. In addition, the fact that each molecule of monoclonal antibody is directed at the target viral antigen can result in an effective increase in the active antibody concentration and
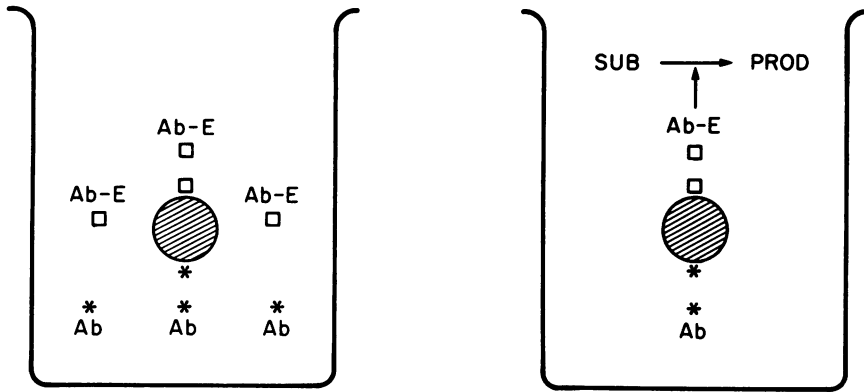
1. Antigen is added to a solid phase coated with antibody directed against one antigenic site (✻Ab). Enzyme labelled antibody directed against a different site on the antigen (☐Ab-E) is then added. This will react with unbound sites on the antigen.

2. Following a washing step to remove unreacted ☐Ab-E substrate is added. This will be converted by bound ☐Ab-E to a measureable product. The amount of product formed will be proportional to the concentration of antigen in the specimen.

FIG. 2. Double determinant enzyme immunoassay.

a subsequent increase in antigen-antibody interaction [30,31]. We have found that, while some monoclonal antibodies can be used efficiently in such formats, many monoclonal antibodies do not have sufficient affinity to be useful in such systems [32,33]. The use of low-affinity antibodies is particularly problematic when the monoclonal antibodies are utilized in solid-phase formats which require multiple incubations, since the dissociation of low-affinity antibodies can occur at each reaction step. Thus many anti-viral monoclonal antibodies which are currently available are not suitable for use in traditional solid-phase immunoassays. For this reason there is a need for the development of assay formats which are not limited in terms of immunoglobulin concentrations and thus can counterbalance lower affinity by the application of the antibodies at higher concentrations. In addition, methods of monoclonal antibody production which would ensure the consistent generation of high-affinity antibodies would represent a significant advance in the application of monoclonal antibodies to the field of microbial diagnostics.

The use of enzymatic molecules as immunoglobulin labels offers a number of advantages in terms of sensitivity, safety, and convenience. However, all enzymes are relatively large molecules (>45,000 daltons). The enzyme-immunoglobulin conjugation procedure thus results in a hybrid molecule which is substantially larger than native immunoglobulin and thus exhibits suboptimal diffusion and binding characteristics [35,36]. For this reason, techniques which utilize the labeling of immunoglobulin with low molecular weight labels in place of macromolecular enzymes result in immunoglobulin conjugates with improved performance characteristics in immunoassay systems for the detection of viral antigens [37]. Such systems are formulated by the labeling of immunoglobulin with a low molecular weight ligand and the subsequent completion of the reaction by the binding of the ligand with an enzymatic molecule which has been modified to specifically recognize the ligand. Since the enzyme-ligand reaction is performed after the antigen-antibody reaction is completed, it does not adversely affect the ability of antibody to bind to antigen in the clinical specimen. While numerous low molecular weight ligands can be utilized in this format, we have found that the use of the avidin-biotin system offers a number of practical advantages

[8,25,37,38]. These advantages include the high affinity of biotin for anti-ligands such as avidin, and the widespread availability of biotin and avidin reagents suitable for use in immunoassay systems. The linkage of biotin to the immunoglobulin can most easily be accomplished by the epsilon amino groups of lysine residues of the immunoglobulin by simple reaction with the n-hydroxy-succinimide ester of biotin. In some cases, however, the labeling can most efficiently be accomplished by linkage through carboxyl [20,39] as sulfhydrl [40] moieties on the immunoglobulin molecule. We have found, for example, that some monoclonal antibodies which cannot be efficiently labeled by reaction with amino groups can be labeled by reaction through these reactive sites. We have found that the avidin-biotin reaction system can be quantitated most efficiently by the binding of the biotinillated antibody with avidin complexed with biotin modified enzyme. The use of an avidin-biotin complex in this way allows for the maximum retention of native enzymatic activity with a resulting optimization of substrate generation and assay sensitivity. In addition, the simple nature of biotin labeling allows for the labeling of a large number of different immunoglobulin molecules in a reproducible fashion. This fact permits the evaluation of a large number of different antibodies directed at the target antigen. Such evaluation is particularly useful in the development of new assay systems when a large number of polyclonal and monoclonal antibodies must be evaluated in order to determine which is optimal for use in the immunoassay system. The avidin-biotin system is thus also useful in the development and optimization of novel assay systems for the detection of new antigen.

An additional advance in the area of viral diagnosis has been an increased knowledge of the early immune response to viral infections in humans. We have found, for example, that viral infections are often accompanied by the presence of viral-specific immune complexes in the infected body site. These complexes are often present in antigen excess and thus contain antigenic sites which are accessible to labeled antibodies. This fact allows for the detection of the specific immune complexes by means of solid-phase immunoassay techniques. Preliminary studies have indicated that the detection of immune complexes can be accomplished in situations in which free antigen cannot be detected. The ability to detect such viral-specific immune complexes thus could markedly expand the applicability of viral diagnostic assays for the diagnosis of such infection.

One problem inherent in all immunoassays is the limitation imposed by the kinetics of antigen-antibody reactions. For this reason it would be desirable to utilize detection systems which do not rely on antigen-antibody interactions. One such system which offers a great deal of potential for viral diagnosis is that of nucleic acid hybridization. Unlike immunoassays, nucleic acid hybridization systems are not limited by the constraints of antigen-antibody reactions [28,29]. Rather, the parameters of reactivity are determined by the association kinetics of nucleotide base pairing. Since each nucleotide can participate in a hydrogen-binding reaction, the kinetics of nucleic acid reassociation is extremely favorable. An additional advantage of nucleic acid hybridizations is that recombinant DNA and other cloning techniques can be utilized to produce large quantities of nucleotide probes with defined degrees of specificity. This fact offers a significant advantage in terms of reagent production and standardization. For these reasons nucleic acid hybridization has been applied to the detection of a number of viral antigens in clinical samples. However, one limitation of nucleic acid detection techniques is that they have relied largely on the use of radioactive isotopes to

achieve nucleotide detection. The use of isotopes is particularly problematic since the isotope which has been most widely used for this purpose, P^{32}, is particularly hazardous and unstable. While non-isotopic nucleic acid labels have been proposed [41,42], available labeling systems involve the use of extensively modified nucleotides which are expensive and difficult to obtain. However, the development of chemical methods for modifying nucleic acids would allow for the use of non-isotopic probes without these limitations. The practical adaptation of nucleic acid hybridization systems for viral diagnosis might represent a marked advance in the area of practical, sensitive viral diagnostics available to the clinical laboratory.

From the above discussion it is clear that there are a number of ways in which currently available microbial assay systems can be improved in order to provide for accurate, practical diagnoses of viral infections under a wide range of clinical and laboratory conditions. While there are numerous ways in which the assay systems can be modified, it is likely that significant advances in microbial diagnosis will derive from developments in the following areas:

1. Improved antigen-antibody interaction formats
2. Increased magnification of the antigen-antibody interaction by means of sensitive indicator systems
3. The optimization of simplified, rapid assay formats
4. Improved assays for the detection of viral-specific immune complexes
5. The adaptation of nucleic acid hybridization techniques to formats that are practical for microbiology laboratories

Our experience with assay systems suggests that it is unlikely that a single assay system will find universal acceptance for the detection of viral pathogens in all possible clinical and laboratory conditions. For example, clinical laboratories at teaching institutions might make good use of assay systems which are sensitive and applicable to a wide range of microorganisms but which might be tedious to perform and which might require some expertise or specialized equipment. On the other hand, medical practitioners will be able to best utilize assay systems which are rapid and simple to perform but which are targeted to microorganisms for which high degrees of sensitivity are not required. The development of a range of assay systems is the most effective way of insuring the development of assay systems which can be applied to the diagnosis of a wide range of viral infections that occur in patients receiving medical care in teaching hospitals, community hospitals, outpatient clinics, doctors' offices, and field stations in the United States and other areas of the world.

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