Chapter 1

Detection of Viral Antigens and Antibodies

Enzyme Immunoassays

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1. APPLICATION OF ENZYME IMMUNOASSAYS IN HUMAN AND ANIMAL VIROLOGY

1.1. Introduction

Serological techniques have been the most widely used means of diagnosing viral diseases for years, but several of their shortcomings have shifted the emphasis toward the direct detection of antigens. Nevertheless, serological testing is still useful if it is impractical or impossible to demonstrate the presence of a viral antigen, e.g., measles virus, togavirus, rubella virus, and Epstein–Barr virus (EBV). Moreover, serological approaches are important in distinguishing between serotypes or isolates. Developments in the design of enzyme immu-

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noassays (EIA) and the production of monoclonal antibodies have permitted the development of techniques that have a specificity approaching or surpassing that required for clinical applications but that do not suffer from the serious drawbacks of classic methods.

The ideal EIA should permit the direct detection of viral antigens and thus offer the possibility of rapid disease management. Unfortunately, this is often not possible because of low concentrations or different serotypes of the viral agents or viremia at certain stages of the disease. Serological testing can be helpful in some situations, such as (1) the case of virus detection but with equivocal interpretation; (2) cases in which virus is suspected but the direct antigen detection is difficult or very time consuming, e.g., enteroviruses, rubella virus, mumps, viral encephalitides, lymphocytic choriomeningitis virus, EBV; (3) heterophile-negative mononucleosis syndromes, i.e., cytomegalovirus (CMV), EBV; and (4) in situations in which the immune status should be established from single sera, i.e., rubella, hepatitis B, varicella zoster.

The EIA has become a useful tool in the research laboratory as well, and virtually all current studies in viral structure and function and viral immunopathogenesis rely on variations of the technique (Mäkelä *et al.*, 1987). The availability and characterization of libraries of monoclonal antibodies directed against specific viral epitopes has come about directly as a result of the application of EIA. Another recent development is the use of chemically defined antigens in immunoassays. Specific viral antigens produced by recombinant DNA technology *in vitro* will have many advantages in different EIA applications in the future. Similarly, synthetic peptides with viral antigenic sites will also be used as reagents in both the basic research and clinical applications of EIA.

Recent developments in recombinant DNA technology permit the use of nucleic acid probes in diagnostic virology. Most probes used in this technique are radiolabeled with [^{32}P]nucleotides. An almost equivalent sensitivity can be achieved by incorporating biotinylated nucleotides into the probes, e.g., by nick translation, and by detecting these probes using succinylated avidin or streptavidin and biotinylated enzymes in a manner similar to the enzyme immunoassays (Langer, 1982). The use of nucleic acid probes obtained by molecular cloning techniques permits the preparation of large amounts of detector molecules for viruses that cannot be cultivated *in vitro*, e.g., hepatitis virus B (HBV) and human papilloma virus. An added advantage of the technique is that, in addition to free nucleic acid, integrated DNA can be detected (Scotto *et al.*, 1983).

Nucleic acid hybridization is particularly useful in the study of herpesviruses can be readily distinguished at low concentrations (Stålhandske and Pettersson, 1982). The technique has also permitted the direct detection of CMV in the urine of immunosuppressed patients (Chou and Merigan, 1983). Similarly,

EBV has been detected at a level of 0.1–0.5 genome equivalents per cell (Brandsma and Miller, 1980; Diaz-Mitoma *et al.*, 1987).

1.2. Humoral Immune Response in Viral Diseases

The humoral immune response to viral infections generally involves the production of immunoglobin G (IgG) antibodies, but with individual variation of the effect of these antibodies on the course and convalescence of the infection (Cradock-Watson *et al.*, 1979). However, in the initial response to a primary viral infection transient IgM antibodies are produced (Schluederberg, 1965). The detection of antiviral IgM antibodies is therefore widely used in diagnosis (Meurman, 1983).

Many exceptions exist to the supposition that a primary viral infection leads to generation of IgM and IgG antibodies and a secondary infection to an IgG antibody population with increased affinity. For example, an IgM response to a primary infection is often absent or low in young children, in immunocompromised persons, in local infections (e.g., respiratory), and in reinfections or reactivation of latent infections. Meurman (1983) noted that IgM tests for confirmed respiratory infections of young children by parainfluenza virus types 1–3, respiratory syncytial virus, and adenovirus were positive in only 25–63% of patients. Moreover, Welliver *et al.* (1980) observed that the IgM response to respiratory syncytial virus infections of 1- to 3-month-old children was much weaker than that of children aged 3–12 months. These viral infections are often too localized to generate systemic immune response (Cranage and Gardner, 1980; Roggendorf *et al.* 1982; Ukkonen *et al.*, 1980). Furthermore, IgM responses are absent in about 25% of influenza virus A or B infections (Goldwater *et al.*, 1982), and similar phenomena have been observed with echovirus, coxsackievirus, and rotavirus infections (McLean *et al.*, 1980; Reiner and Wecker, 1981). This observation may reflect the fact that these patients earlier had a primary infection with a related virus, and the measured response is therefore secondary in nature.

Although the absence of an IgM response after rubella infections has been regarded as indicative of reinfection (Boué *et al.*, 1971), an IgM response has been recorded in about 20% of vaccinated subjects reinfected with live rubella virus (Harcourt *et al.*, 1980). The IgM antibody response to reactivation of latent infections of herpesvirus types 1 and 2 (HSV-1 and HSV-2) is rare, but frequent (70–80%) in cases of herpesvirus zoster (HSV) (Meurman, 1983). Reactivation of CMV sometimes results in high IgM responses (Cappel *et al.*, 1978), although low values have also been reported (Kangro, 1980; van Loon *et al.*, 1981).

Temporal variations in the IgM antibody response may also lead to difficulties in interpreting the results of IgM tests. Complicated infections often result in

a prolonged IgM response (Burke and Nisalak, 1982); for example, a rubella virus IgM response of up to 4 years has been observed (Stallman *et al.*, 1974). Subacute sclerosing panencephalitis patients with chronic measles infection very rarely have IgM antibodies (Ziola *et al.*, 1979). After renal transplantation in immunosuppressed patients, CMV, and BK papovavirus are often reactivated followed by a prolonged IgM response (Cappel *et al.*, 1978; Flower, 1977). Patients with chronic hepatitis virus B (HBV) or healthy carriers also frequently have a prolonged IgM antibody response (Roggendorf *et al.*, 1981).

It should be noted that the detection of these IgM responses may simply arise because of the high sensitivity of the assays compared with less sensitive tests. The value of IgM tests may also be decreased for infections with related serotypes of viruses. Viruses with uniform antigenicity that produce long-lasting immunity are characterized by a constant and transient IgM response (Meurman, 1983), However, viruses for which several strains or serotypes exist may lead to an unpredictable IgM response, e.g., absence of IgM response, or false-positive reactions. The increased specificity and sensitivity of enzyme immunoassays can have a profound impact on the determination of specific IgM antibodies for heterogeneous groups of viruses such as enteroviruses, togaviruses, para-influenza virus, and adenoviruses.

In recent years, EIA have permitted the study of the response of subclasses of IgG antibodies to viral infections. These subclasses differ in their biological properties, and subclass profiles of antibodies may indicate the state of infection. IgG1 is the major subclass; antibodies to viruses are usually found in this subclass, although subclass IgG3 antibodies are also frequently found (Linde, 1983; Morell *et al.*, 1983; Sundqvist *et al.*, 1984). As a whole, IgG2, the second subclass, appears mainly to contain antibodies to polysaccharides, e.g., lipo-polysaccharides of bacteria. Subclass IgG3, which does not react with protein A, has a rapid turnover and is the most active subclass in activating the C1 component of complement; it is frequently associated with recurrent illnesses (Gilljam *et al.*, 1985). Subclass IgG4 antibodies have been detected after herpesvirus infections. Interestingly, IgG4 responses to HSV are common, but for CMV and varicella zoster virus (VZV), a familial relationship has been observed (Gilljam *et al.*, 1985; Vejtorp *et al.*, 1980), indicating that IgG4 responses are related to allergic disorders that may have a genetic component. The principles of class-capture assays to detect antibodies belonging to specific classes or subclasses are shown in Fig. 1.

1.3. Technical Aspects of IgM Antibody Assays

Early methods still widely used to distinguish IgM from IgG antibodies were based on the physical separation of IgM from IgG by sucrose-density gradient fractionation, gel filtration, affinity or ion-exchange chromatography.

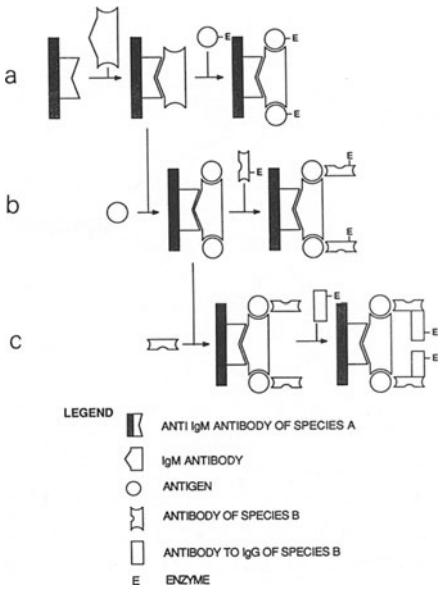

FIGURE 1. Principles of class-capture assays. The anti-immunoglobulin class antibody (e.g., IgG or IgM) is adsorbed on the solid phase and will trap molecules of that class. If antibodies are contained within this Ig class, antigen added subsequently will also be immobilized (a). This antigen can then be detected either directly (b) or indirectly (c).

Alternatively, IgM molecules can be selectively dissociated by thiols to produce subunits with lower activity. However, to produce significantly different titers for untreated and treated serum, at least 75% of total antibodies needs to be of the IgM class. However, only a few viruses cause such marked IgM immune responses, e.g., tick-borne encephalitis (Kunz and Hofman, 1971). Nevertheless, this method can be used in conjunction with physical separation methods.

The basic EIA for IgM antibodies employs viral antigens captured on a solid phase. Specific viral antibodies to all subclasses bind to this antigen in the assay. Bound antibodies of a specific subclass are detected by class-specific enzyme-labeled antibodies. If the amount of specific IgM antibody is low compared with IgG antibody, the positive signal in the IgM test is low. Another problem in this type of direct assay is interference by rheumatoid factors. These problems can be reduced using reversed assays in which all the IgM antibodies are first captured on a solid phase and the specificity then determined by use of specific antigens (Figure 2). Duermeyer and van der Veen (1978) first reported such a class-capture EIA (Fig. 1) that avoided the problems mentioned. A drawback is that the sensitivity of IgM detection is influenced by the fraction of specific IgM antibodies in the total IgM pool (Heinz *et al.*, 1981).

Nonspecific adsorption of IgM to a solid phase seems to be higher than that of IgG (Meurman, 1983) because of the law of mass action and the physical properties of IgM. Rheumatoid factors (RF) have a steep dose–response curve (Meurman and Ziola, 1978), and dilution is one way of decreasing their inter-

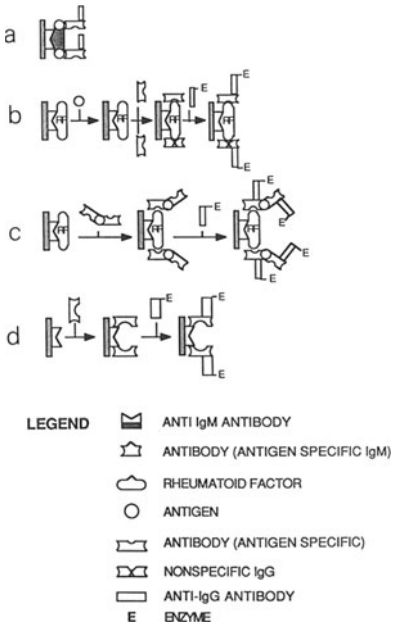

FIGURE 2. Class-capture assay (a) and possible causes of nonspecific interactions (b,c,d). Anti-immunoglobin M (IgM) assays may cause the nonspecific adsorption of rheumatoid factors (RF) that adsorb IgG without the presence of antigen (b) or immune complexes (c). Moreover, nonspecific protein–protein interactions (d) may cause background staining.

ference. RF are produced in patients with autoimmune and infectious disease. Pregnancy is also often accompanied by the activation of RFs (Meurman, 1978). Current methods to decrease RF interference in immunoassays involves the adsorption of specimens with heat-aggregated IgG (Kurstak *et al.*, 1977) or glutaraldehyde (Krishna *et al.*, 1980), with latex particles covered with IgG (Vejtorp *et al.*, 1980), or excess normal IgG (Milan and Stigbrand, 1981). None of these methods is entirely satisfactory. The use of $F(ab')_2$ instead of intact IgG is, however, often effective in reducing nonspecific reactions. The principles employed in some frequently used EIA are shown in Fig. 3.

1.4. Viral Antigen Detection in Clinical Specimens

A large number of virus particles are known to be secreted during the acute phase of viral diseases. It was shown that viral antigens can be detected in nasopharyngeal cells by the fluorescent antibody technique (Gardner and McQuillin, 1968). Electron microscopic observation that patients with rotavirus infection excrete a large number of virus particles (Flewett *et al.*, 1974) led to the first successful attempts to detect viral antigens in clinical specimens by immunoassay (Middleton *et al.*, 1977; Yolken *et al.*, 1977). It was shown later that a number of acute viral diseases could be efficiently diagnosed by the demonstration of virus antigens in clinical specimens (Yolken, 1982a).

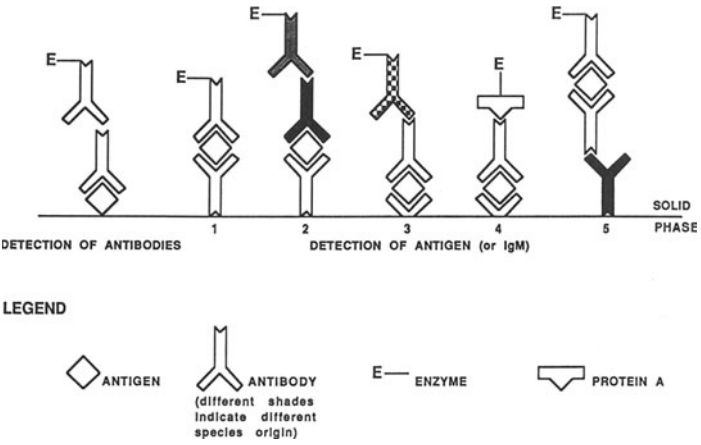

FIGURE 3. Frequently used enzyme immunoassays for the detection of antibodies, antigen (1–4), or antibodies belonging to specific immunoglobulin classes (5). The molecules to be detected are always found in the second layer.

Different technical variations of the antigen detection method have been described (Fig. 3). Provided that specific, high-titered antisera are available, the establishment of a reliable antigen assay for detection of virus antigen is technically feasible. The threshold sensitivity of the assay system depends mainly on the quality of the antiserum and the enzyme used. Antigen concentration of ~1 ng/ml has been reached in most of the antigen assays but it is theoretically possible to measure attomoles (10^{-18} M) of viral antigens by EIA (Richman *et al.*, 1984). Such ultrasensitive tests will ultimately be developed, but even the present EIA are sensitive enough for the diagnosis of most acute viral infections.

The major problems in antigen-detection systems may be in collection of the specimens. The best method for obtaining specimens from respiratory infections is the collection of nasopharyngeal secretions. Collection of stool specimens from gastroenteritis patients does not require any special technique. As the virus secretion is short-lived during an acute viral infection, timing of the specimen collection is critical. Information about the period of antigen excretion in different viral diseases is fragmentary. It can be safely assumed that during the early phases of the disease the likelihood for antigen-positive specimens is high. Therefore, the specimens should be collected as early as possible during the infection. Another problem in specimens collected late during viral infection is the formation of immune complexes between the antigen and antibodies. Detection of virus antigens in such complexes cannot be performed by ordinary antigen assays but requires laborious separation techniques (Lund and Salmi, 1982) or capture EIA designed to detect antigen–antibody complexes (Crouch *et al.*, 1985).

1.5. Detection by EIA of Human and Animal Virus Infections

1.5.1. Hepatitis

The presence of hepatitis B surface antigen (HBsAg) in blood is frequently used as an indicator of HBV infection and concentrations may range from 0.001 to 50 μg/ml. HBsAg is, however, a poor marker for infections, as serum may transmit HBV in the absence of detectable HBsAg. In model situations, DNA probes have enhanced sensitivities and may be the only method of detecting HBV infection (Feinman, 1985; Harrison *et al.*, 1983; Thomas *et al.*, 1985).

Detection of a weak anti-hepatitis B core (HBc) IgM response in HBsAg-positive patients cannot always be considered a definitive marker of recent infection. However, quantitation of HBc IgM in HBsAg carriers using class-capture EIA increases the clinical value of the test and permits differentiation between acute HBV infections and jaundice due to other causes (Kryger, 1985). Anti-HBc IgM class capture EIA tests are important, since HBsAg carriers generally exhibit very high total antibody titers for anti HBc.

Papaevangelou *et al.* (1984) showed that the diagnostic value of anti-HBc in areas in which the prevalence of HBV is high is important because complex situations arising from other forms of acute liver injury may complicate the diagnosis of a patient with chronic HBV liver disease.

Hepatitis A virus (HAV) can be detected in cell culture by electron microscopy with immunoperoxidase staining (Asher *et al.*, 1987), a technique that is particularly useful, considering the difficulty in obtaining suitable culture systems for this virus.

HDV (delta agent, δ) can be detected by the immunoperoxidase method (Recchia *et al.*, 1981) and by EIA for determination of total anti-HDV antibodies (Shattock and Morgan, 1984) or IgM using a class-capture assay (Smedile *et al.*, 1982). The potential importance of HDV has been stressed by Smedile *et al.* (1982), who found the incidence of HDV markers higher in patients with fulminant hepatitis than in those with benign hepatitis. The use of antigen derived from detergent-treated serum increases the sensitivity of the EIA for HDV (Shattock and Morgan, 1984).

1.5.2. Viral Gastroenteritis

Viral gastroenteritis is common worldwide, its prevalence being second only to that of respiratory illnesses (Smedile *et al.*, 1982). Its clinical features vary and, after a rapid onset, include nausea, vomiting, abdominal cramps, headache, anorexia, myalgia, and malaise. The maladsorption triggered by gastroenteritis-associated viruses may increase morbidity and mortality in poorly nourished populations.

Rotaviruses infect the gastrointestinal (GI) tracts of many species, including man, and produce clinical and serological effects that are age dependent. Subgroup specificity of the particular rotaviruses is determined by the major inner capsid protein and the serotype specificity by an outer capsid glycoprotein (Kalica *et al.*, 1981). Cultivation of human rotaviruses in the laboratory is not satisfactory for diagnostic purposes (low isolation rates and the need for several passages). Solid-phase EIA for rotavirus antigen have proved useful in this respect (Yolken, 1982b); however, nonspecific reactions frequently occurred in early studies (Krause *et al.*, 1983). Nevertheless, various modifications, such as the use of chelating agents, protease inhibitors, pH neutralization, and the inclusion of normal serum, greatly improved the reliability of these diagnostic tests (Beards *et al.*, 1984; Hammond *et al.*, 1982; Hogg and Davidson, 1982; Hovi *et al.*, 1982). The availability of a monoclonal antibody to the common rotavirus antigen has increased the sensitivity and specificity of commercially available tests.

Another important gastroenteritis virus is the Norwalk virus. The low shedding of the Norwalk virus has so far hampered its characterization and the preparation of a hyperimmune serum. Infantile gastroenteritis is often caused by adenovirus serotypes 40 and 41 (Brandt, 1983; De Jong *et al.*, 1983), which are readily identifiable by EIA.

A radioimmunoassay (RIA) has been developed to Norwalk agent and has been proved more efficient than immunoelectron microscopy (Blacklow *et al.*, 1979) for antigen detection. Similarly, a RIA-blocking test has been used for serological investigations into Norwalk virus infections. Similar studies should be possible with EIA.

Using a capture EIA assay, it was possible to study the persistent shedding of bovine enteric coronavirus antigen–antibody complexes in clinically normal cows over extended periods (Crouch *et al.*, 1985). Similarly, EIA can be used to detect other GI infections, such as astroviruses, caliciviruses, toroviruses, and other enteric infections, that may be important in causing GI infections in man and animals (Ashley *et al.*, 1978; Spratt *et al.*, 1978).

1.5.3. Herpesviruses

Numerous serological tests and other procedures have been developed for the specific detection of antibodies to HSV-1 and HSV-2 in human sera, e.g., neutralization kinetics, microneutralization, indirect hemagglutination, immune lysis of infected cells, immunofluorescence, immunoperoxidase assay, solid-phase RIA, immunoelectrophoresis, and polyacrylamide gel electrophoresis (PAGE) after immunoprecipitation (Hampar *et al.*, 1985; Kurstak *et al.*, 1977; Kurstak, 1986; Kurstak *et al.*, 1984a; Kurstak *et al.*, 1984b). Microneutralization is most frequently used in seroepidemiological studies (Pauls and Dowdle,

1967; Rawls *et al.*, 1979), but EIA methods are faster and easier to perform (Coleman *et al.*, 1983; Katz *et al.*, 1986; Vestergaard, 1982). Hampar *et al.* (1985) developed a modified EIA test that uses as target antigens highly immunogenic glycoproteins purified with monoclonal antibodies; the method uses heterologously infected cell extracts for the absorption (30 min) of cross-reacting antibodies from test sera. The index of agreement with microneutralization is close to 100%. Monoclonal antibodies have been used in immune affinity chromatography to purify each of the three major glycoproteins of varicella zoster virus (VZV) and bovine herpesvirus 2 (BHV-2) for subsequent use as solid-phase reagents in EIA for detection of VZV specific antibodies (Keller *et al.*, 1986).

Using purified bovine herpesvirus-1 (BHV-1) glycoproteins, a solid-phase EIA has been established to test the kinetics of the development of the immune response as well as the specificity of the immune response to the individual BHV-1 glycoproteins (Babiuk *et al.*, 1987; van Drunen Littel-van den Hurk and Babiuk, 1986). These assays in addition to testing the efficacy of specific glycoproteins and immunogens also have the potential of being used to differentiate animals which are immunized with individual glycoproteins from animals immunized with conventional viruses or those exposed to field strains of virus. This differentiation is becoming important as producers and countries try to eliminate herpesvirus infections from their herds. A similar approach is being used in the control and eradication of pseudorabies virus (PRV) in pigs. A subunit vaccine is being marketed in conjunction with a card EIA. Using this approach, producers test animals for the presence of antibody to the glycoprotein being used in the vaccine, as well as to a second major glycoprotein not present in the vaccine. All animals that possess antibodies to the glycoprotein that is not in the vaccine are considered to be potential carriers of latent pseudorabies virus. These animals are then eliminated from the herd and replaced with animals that are seronegative to that specific test glycoprotein. Within a short time, it is hoped that such an approach would eliminate all latent carriers of PRV. Although a similar test has been conducted with BHV-1 under laboratory conditions, to the authors' knowledge, this is not being developed commercially.

It has been suggested that IgE contributes to the pathogenesis of herpesvirus infection (Ida *et al.*, 1983); however, there have been few reports of the production and significance of antiviral IgE antibodies. This may be because of difficulties arising from competitive inhibition of antiviral IgE binding to antigenic determinants caused by the presence of excess IgM, IgA, or IgG antibodies. To circumvent this problem, van Loon *et al.* (1981) devised a sensitive direct EIA test based on the antibody-capture principle (sequence: affinity-purified, anti-human IgE-coated microtitration plates; sample with IgE antibodies; peroxidase-labeled viral antigens). This method may become an important tool for investigating the role played by the IgE response in immunological defense and viral pathogenesis.

Cytomegalovirus infections are serious if recurrent, particularly in immunocompromised patients (Whelchel *et al.*, 1979; van Loon *et al.*, 1987). Several factors prevent EIA detection of CMV in fresh urine by binding to the virus, masking its antigenic determinants (McKeating *et al.*, 1986; El-Mekki *et al.*, 1987). A competitive EIA method was recently developed for detecting CMV antibody in the sera of cardiac transplant patients. The method is five times more sensitive than the complement-fixation test and twice as sensitive as indirect EIA (Wreghitt *et al.*, 1986). Ida *et al.* (1983) obtained evidence that the IgE response to herpesvirus infection is important in patients with decreased numbers of T-suppressor cells because of immunosuppressive therapy.

1.5.4. Pestiviruses

Maternal rubella is transmitted to the fetus by way of viremia, particularly during early pregnancy (Miller *et al.*, 1982), resulting in an infection rate of 80–90% for the placenta and 60–70% for the fetus. In about 10% of cases, spontaneous abortion results and in 25–35% there is malformation of the fetus.

Several problems are encountered in the serological diagnosis of rubella: accidental rubella vaccination during pregnancy, reinfection of previously vaccinated women during pregnancy, acute rubella infection or contact with infected individuals, and rubella immunity during pregnancy.

Antibodies to rubella are present in the newborn up to the age of about 6 months. Cell-mediated immunity is also important in the control of rubella infections. The level of IgM antibodies declines rapidly after the onset of symptoms. The detection of rubella specific IgM antibodies indicates recent infection by rubella. For standard hemagglutination inhibition tests, serum fractionation is necessary but is impractical for large-scale screening. Considerable research has gone into developing class capture assays of the EIA type for rubella (Bellamy *et al.*, 1986; Isaac and Payne, 1982; Vejtorp *et al.*, 1980). Physical separation of IgG from IgM antibodies need not be carried out and only 5 μl of sample suffices. In this respect, the $F(ab')_2$ conjugates have proved particularly reliable and sensitive probes.

One virus that is a problem for the cattle industry due to its ability to infect animals and cause disease, as well as in anyone working with tissue culture is the bovine viral diarrhea (BVD) virus. This virus can often infect immunologically naive animals; if the animal is pregnant, the virus can be transferred across the placenta, infecting the fetus. Depending on the stage of gestation, a number of scenarios may occur. First, the animal may be infected with a virus and become immunotolerant to the virus. This generally occurs if animals are infected before the 130th day of gestation. Second, the animal may develop antibodies and clear the virus before birth. This occurs if the animal is infected after 130 days of gestation. The animals infected prior to 130 days of gestation are born and are

persistent shedders of BVD virus and are immunotolerant to the virus. To prevent continued infection and spread of the virus, it is important to identify these carriers by detecting the presence of virus in the blood. This can be done either by EIA or fluorescent antibody tests (Bielefeldt Ohmann *et al.*, 1982; Bolin *et al.*, 1985; McClurkin *et al.*, 1984). If animals are killed during pregnancy and fetal bovine serum (FBS) is collected for use in tissue culture, the serum may contain either BVD or virus–antibody complexes. FBS containing either BVD or BVD antibody, or both, is unsatisfactory as a component of cell-culture media for growing viruses, for research, or for vaccine production. Noncytopathic BVD present in the serum may infect cell cultures, resulting in viral interference with replication of other viruses or alter the kinetics of viral protein synthesis. There are EIA and competitive blocking EIA for detecting either BVD antibodies or antigen in the serum (Katz and Hanson, 1987).

1.5.5. Measles

In many countries, the prevalence of measles has decreased dramatically since the introduction of measles immunization (Hinman *et al.*, 1983; Larke, 1983; Peradze and Smorodintsev, 1983; Sejda, 1983). Nevertheless, a diagnostic screening test to identify nonimmune persons is important because of the severe complications that can occur after infection with measles virus (Morgan and Rapp, 1977), and the occurrence of measles virus outbreaks in supposedly protected populations (Marusyk, 1984). Hemagglutination inhibition tests have been widely used for this purpose but are inconvenient, as sera should be pretreated to remove nonspecific inhibitors or agglutinins that would otherwise influence the reliability or specificity of the test.

Measles may be associated with complications such as prolonged diarrhea, encephalitis, pneumonia, otitis media, and sometimes subacute sclerosing panencephalitis (SSPE). The global case fatality rate in developing countries has been estimated to approach 2%, i.e., 1.5 million deaths per year (Marusyk, 1984).

Enzyme immunoassays are as sensitive as other serological techniques in detecting seroconversion to measles virus (Rapicetta *et al.*, 1983; Tuokko and Salmi, 1983). A convenient and flexible EIA method for detecting antibody to measles antigen was introduced by Rice *et al.* (1983). In this approach, the wells of microtitration plates are coated with infected cells. Prepared in this way, the plates can be stored desiccated for months at room temperature; the major viral epitopes are preserved and can be detected by conventional EIA procedures. Measles-capture assays for IgM using monoclonal antibodies are useful and produce little or no background values (Forghani *et al.*, 1983; Kramer and Cremer, 1948; Tuokko, 1984).

Forghani *et al.* (1982) produced monoclonal antibodies to human IgM that

were evaluated in capture-class assays for measles and rubella IgM antibodies. This approach avoided cross-reactivity with IgG and eliminated the need to include antibody-negative human sera in the diluents for test reagents (Isaac and Payne, 1982). False-positive reactivity caused by the presence of rheumatoid factors was virtually eliminated using glutaraldehyde-aggregated IgG as an absorbent. Latex agglutination tests are poor indicators of rheumatoid factors and give high levels of false-positive reactivity.

1.5.6. Infections of the Respiratory Tract

Most infections of the upper respiratory tract are caused by viruses, but bacteria may account for up to one third of such infections. Conventional hemagglutin inhibition and neuraminidase inhibition tests for influenza antibodies are relatively insensitive (Turner *et al.*, 1982). However, EIA methods are much more sensitive and permit the assay of antibodies in nasal washings. Murphy *et al.* (1981) developed an EIA test for hemagglutinin instead of for the whole virus, whereas Khan *et al.* (1982) reported an EIA test for neuraminidase-specific antibodies. A pitfall in the determination of titers for influenza viruses was pointed out by Madore *et al.* (1983), who observed that dose–response curves could be displaced upward, reflecting an increase in antibody affinity, without a change in the end point (steeper curves). Differentiation of influenza virus strains requires the use of the constituent proteins rather than of whole virus (Al-Kaissi and Mostratos, 1983).

Parainfluenza viruses are associated with many common respiratory illnesses of children. The main problems in standard methods for the determination of parainfluenza viruses is the rapid loss of infectivity and the high cost of cell-culture assay systems. Immune responses may take several weeks before a detectable antibody titer is present. In contrast to aspiration with a mucous extractor (through the nostrils), the use of nasopharyngeal washings gives low yields of parainfluenza antigen. Cross-reactivity between antibodies to parainfluenza virus types 1 and 3 as well as with antibodies to mumps virus can be significant (Julkunen, 1984). Live virus is not required for EIA.

Respiratory syncytial virus (RSV) is also commonly diagnosed by cell-culture techniques. EIA have been introduced and are an important alternative (Sarkkinen *et al.*, 1981). Compared with cell-culture methods, the EIA has a sensitivity of more than 90% and a specificity of more than 96% (Flanders *et al.*, 1986). EIA methods have also been used to detect coronaviruses successfully (Kraaijeveld *et al.*, 190; Macnaughton *et al.*, 1983) at a level of 10^5 particles/ml. Adenovirus antigen can also be detected in nasopharyngeal specimens from patients with respiratory tract infections (Sarkkinen *et al.*, 1981).

An EIA that detects rhinovirus-specific antibody in human sera and unconcentrated nasal secretions is 100- to 1000-fold more sensitive than standard neutralization assays (Barclay and Al-Nakib, 1987).

1.5.7. Rabies

The classic method for detecting neutralizing antibodies for rabies is seroneutralization using an *in vivo* mouse system. However, the results obtained are variable, and the method is time consuming (up to 21 days). Wiktor *et al.* (1973) improved the reproducibility and speed of the method by using tissue-culture and inhibition immunofluorescence, while Atanasiu and Perrin (1979) developed an immunoenzymatic micromethod for the titration of antirabies antibodies using as antigens either the rabies virus or a constituent glycoprotein. Significant results were obtained within a few hours (Sureau *et al.*, 1982).

Both rabies vaccine and laboratory-adapted rabies virus replicate in cell culture to give high titers. However, wild strains of the virus grow poorly in cell cultures, and it is difficult to produce the amount of virus required for large scale screening. Smith *et al.* (1984) developed a procedure in which cells infected with γ-irradiated rabies virus rather than the purified virus are used as target antigens. A single freeze-thawing cycle permits reaction of the antibody with both internal and external (nucleocapsid and membrane) viral antigens.

1.5.8. Arboviruses and Viral Fevers

Attempts to isolate arboviruses from patients or animals are rarely successful, and the diagnosis usually depends on serological testing. Although complement fixation, hemagglutination inhibition, and neutralization are sometimes useful in this respect, each has important disadvantages (Calisher and Poland, 1980). Specific antibody-capture immunoassays have proved useful for detecting an immune response to arboviruses, e.g., Japanese encephalitis (Burke *et al.*, 1982; Burke and Nisalak, 1982; Chang *et al.*, 1984; Konishi and Yamaoka, 1982; Zhang *et al.*, 1979; Roggendorf *et al.*, 1981), St. Louis encephalitis (Monath *et al.*, 1984; Wolff *et al.*, 1981), and yellow fever (Duebel *et al.*, 1983).

Kurstak *et al.* (1980) detected arbovirus antigens in infected cells using the immunoperoxidase method. The use of this method permitted McLean *et al.* (1979) to demonstrate the intracytoplasmic replication of California encephalitis virus in both domestic and wild mosquitoes.

An illustrative example is the detection of Lassa virus antigens and Lassa virus-specific antibodies. Lassa fever is a severe, often fatal, human disease of considerable importance in some regions of West Africa. Almost all patients with Lassa fever are viremic upon admission, but tests designed to determine Lassa virus infectivity require 2–7 days, a high biological containment laboratory, and tissue-culture facilities. Niklasson *et al.* (1984) developed an EIA using β-propiolactone-inactivated sera that provides a definitive diagnosis within a few hours.

1.5.10. Acquired Immune Deficiency Syndrome

The need for a rapid and accurate diagnosis of acquired immune deficiency syndrome (AIDS) by a reliable and sensitive test has resulted in a burst of commercially available enzyme-linked immunosorbent assays (ELISA) for antibodies to HIV-1 virus (Cooper *et al.*, 1978; Evans *et al.*, 1987; Oldham *et al.*, 1987). The tests are based on HIV-1 antigen attached to a solid-phase support. The antibodies in human specimens bind to the antigen-coated solid phase and are visualized by enzyme-tagged antibodies to human Ig. Most tests for HIV antibodies require reading by a photometer, but simpler bedside EIA requiring only visual observation have also been described (Ivo dos Santos *et al.*, 1987).

Detection of antibodies in serum or cerebrospinal fluid (CSF) specimens (Goudsmit *et al.*, 1986) is the only practical large-scale routine method for definite viral diagnosis. HIV antigens can also be detected by immunoassays (Higgins *et al.*, 1986), but the amount of antigen is too low for direct measurement of these antigens in clinical specimens from AIDS patients. Although the reliability of the EIA for HIV antibodies is good, a small percentage of the test results should be confirmed by Western blotting on electrophoresed virus polypeptides. The strain variation of HIV-1 virus is not an obstacle to antibody determination, as some of the strong antigenic determinants are conserved. A more serious problem in HIV-antibody determinations is the existence of one or more antigenically distinct HIV viruses, such as HIV-2 (Clavel *et al.*, 1986). Low-level cross-reactivity between these viruses may be seen in EIA with the HIV-1 antigen, but sensitive antibody tests for these viruses require a homologous antigen on the solid phase (Brun-Vezinet *et al.*, 1987).

The antigens used in the first tests for HIV antibodies were produced in tissue culture. Most tests still employ purified HIV virions on solid phase, but some newer tests are based on antigens produced by recombinant DNA technology or, recently, on synthetic peptides with predicted sequences of HIV proteins (Burke *et al.*, 1978; Shoeman *et al.*, 1987). Antibody response against both the internal (p24 and p55) and envelope (gp41, gp120) proteins occurs in infected persons, but the development of all these antibodies is slow, beginning 1–2 months after the infection (Cooper *et al.*, 1987; Gaines *et al.*, 1987). The best response occurs against the internal components and the transmembrane envelope protein gp41 (Schüpach *et al.*, 1985). The peak immune response may be relatively weak in some patients or may decline when the disease progresses. The optimal type of virus antigen to be employed in the diagnostic EIA for HIV antibodies has not been determined, but it is likely that a combination or more than one polypeptide or synthetic peptide will give the most reliable results.

As the HIV antigens produced in T-cell lines contain cellular material, particularly molecules with function in the immune system (Hozie *et al.*, 1987), autoreactivity against HLA antigens and other molecules on lymphocytes causes

false-positive reactions. Patients with a history of multiple blood transfusions or multiple pregnancies and patients with organ transplants or past dialysis treatments are especially susceptible to development of antilymphocyte antibodies (DeSanto *et al.*, 1987). Variations in the virus antigens used in the tests also have an effect on the false-positive reactions (Wartick *et al.*, 1987). The problems caused by lymphocyte antigens in the viral preparations can probably be avoided when recombinant proteins or synthetic peptides are employed in the EIA for HIV antibodies.

The false-positive results in the studies of a larger number of healthy populations is less than 1%. As the EIA for HIV antibodies are made to be very sensitive, false-negative results are rare. However, EIA antibody tests may give negative results in HIV-infected persons in two principal circumstances. First, antibodies against HIV develop slowly (Gaines *et al.*, 1987), and specimens taken only a few weeks after infection may remain negative. Second, HIV antibodies may also disappear at later stages of the disease (Weber *et al.*, 1987).

In addition to using the EIA in detecting oncogenic viruses in man, EIA are used extensively in detecting RNA tumor viruses in animals. These are used both to detect free virus, virus antigen–antibody complexes, and viral antigen in the serum. There is some difference of opinion on the relative accuracy of EIA versus immunofluorescent (IFA) procedures (Lutz *et al.*, 1983). It must be emphasized that when both tests are performed properly, the correlation between them is more than 95% (Lutz *et al.*, 1980). The 5% discrepancy arises in that EIA detects cats that are persistently antigenemic but IFA negative. These are not false-positive EIA results, as cats in this category often have high antibody titers to FELV and OCMA. In some of these animals, infectious FELV was found in several organs, including salivary gland and urinary bladder. These cats belong to a category of silent shedders that are not detected by the IFA procedure. In addition to being more sensitive and identifying a category of cats that are negative by IFA, the advantage of EIA is its greater sensitivity and the ease with which it can be conducted. EIA also lends itself to use in a clinician's office. A disadvantage of the procedure is that false-positive results may result when the test is improperly done. Most false-positive results are associated with improper or too rapid washing of the reaction wells at the end of the incubation steps, and with the use of whole blood or badly hemolyzed serum. In the case of hemolyzed serum, it is very difficult to wash away, resulting in false-positive results. Thus, if clinicians are to use this procedure, they must be aware of the problems associated with the assay.

Results with EIA using tears or saliva give similar results to blood tests (Hawkins *et al.*, 1986). If this is the case, it may reduce some of the false-negative results seen with improper washing procedures. Although it is often not a problem to detect gp27 in feline leukemia virus (FLV) cats by EIA, a micro-EIA has also been developed to investigate immune complexes present in the

blood of leukemic cats (Tuomari *et al.*, 1984). This assay uses a solid-phase C1q protein A peroxidase EIA to quantitate circulating immune complexes during the course of infection. Increases in circulating immune complexes occur early in the infection and are often cleared following recovery. A persistent elevation of circulating immune complexes is an indication of a cat with persistent viremia.

Bovine leukemia virus (BLV), similar to HIV, has a long incubation period with clinical signs occurring late in the disease. One of the prominent early features of infection with BLV is the presence of antibody against unglycosylated components, mainly the major internal protein p24 and against virus envelope glycoproteins gp51 and gp30. These antibodies are detectable within 2 or 3 weeks after experimental infection and constitute the earliest possible indication of BLV infection (Portelle *et al.*, 1983, 1984). To reduce the horizontal spread of virus between animals within a herd, and for elimination of the disease from herds, it is important to have a method for early detection of infection (Manz *et al.*, 1981). Both direct binding of BLV virus to microtiter plates or, more recently, the development of monoclonal antibodies to individual glycoproteins can be used as a capture EIA (Bruck *et al.*, 1981). To date, the BLV gp51 EIA appears to be the most sensitive method for early detection of BLV antibodies.

2. APPLICATION OF ENZYME IMMUNOASSAYS IN PLANT VIROLOGY

2.1. Introduction

The EIA technique was used to detect plant viruses for the first time by Voller *et al.* (1976). The following year, Clark and Adams (1977) described in detail the double antibody sandwich (DAS) form of EIA, a format that has become the one most commonly used by plant virologists. In this method, the wells of a microtiter plate are first coated with antivirus globulins, and the virus in the test sample is then trapped by the solid-phase antibody. The presence of virus is demonstrated by an enzyme-labeled antivirus conjugate prepared from the same antiserum used for coating the plate (procedure 4, Table I). In spite of its great popularity with plant virologists, this test suffers from two limitations: (1) it requires the preparation of a different antivirus enzyme conjugate for each virus to be tested; and (2) it is extremely serotype specific, which means that enzyme conjugates prepared with antibodies against one virus strain often fail to react with other strains of the same virus species (Koenig, 1978; Lister and Rochow, 1979; Van Regenmortel and Burckard, 1980; Rybicki and Von Wechmar, 1981; Devergne *et al.*, 1981; Koenig and Paul, 1982; Wetter *et al.*, 1984).

Applications of DAS–EIA in plant virology have been described in several

Table I
Types of EIA Used in Plant Virology[a]

Procedure	Types of assays
1	AG, Ab^{R}-E
2	AG, Ab^{R}, $antiR^{G}$-E
3	Ag, Ab^{R}, protein A–E
4	Ab^{R}, Ag, Ab^{R}-E
5	Ab^{C}, Ag, Ab^{R}, $antiR^{G}$-E
6	$F(ab')^{R}$, Ag, Ab^{R}, $antiFcR^{G}$-E
7	$F(ab')^{R}$, Ag, Ab^{R}, protein A–E
8	Ab^{R}, Ag, MAb^{M}, $antiM^{R}$-E
9	Ag, Ab^{M}, $antiM^{R}$, $antiR^{G}$-E
10	$AntiM^{R}$, MAb^{M}, Ag, Ab^{C}, $antiC^{R}$-E
11	Ab^{C}, Ag, Ab^{M}, $antiM^{R}$, $antiR^{G}$-E
12	MAb^{M}, Ag, MAb^{M}-Bio, Avi-E
13	Protein A, Ab^{R}, Ag, Ab^{R}, protein A–E

[a]Ag, antigen; Ab, antibody; R, rabbit; G, goat; C, chicken; E, enzyme; MAb, monoclonal antibody; M, mouse; Bio, biotin; Avi, avidin; F(ab'), fragment of immunoglobulin.

reviews (Bar-Joseph and Garnsey, 1981; Clark, 1981; Torrance and Jones, 1981; Clark and Bar-Joseph, 1984); this topic is not reviewed again here. Instead, this section concentrates on the other EIA formats used with plant viruses. Attention is also given to some recent methodological advances.

2.2. Types of EIA Used in Plant Virology

Enzyme immunoassay methods can be divided into direct and indirect procedures. In the first type of procedure, the antivirus antibody is itself labeled with an enzyme, while in the second type, the enzyme conjugate is an anti-Ig reagent. This points to two advantages of indirect EIA methods: (1) a single enzyme conjugate, such as a goat antirabbit globulin conjugate can be used to detect any number of different viruses, eliminating the need for separate conjugates for each virus system; and (2) since antiglobulin enzyme conjugates are commercially available at very competitive prices, the need for preparing conjugates is eliminated.

Some of the EIA methods used with plant viruses are listed in Table I. The simplest forms are procedures 1–3, in which the viral antigen is adsorbed directly to the solid phase. In many cases, there is enough viral antigen present in crude leaf extracts to make it possible to identify infected plants by incubating crude plant juice directly in the wells (Lommel *et al.*, 1982; Mowat and Dawson, 1987). Leaf extracts can be prepared with pH 9.6 carbonate buffer, with pH 7.4

phosphate-buffered saline containing 0.05% Tween-20 and 2% polyvinylpyrollidone or simply with water (Lommel *et al.*, 1982; Ehlers and Paul, 1984). The adsorbed antigen can be detected with an enzyme-labeled antivirus conjugate (procedure 1), indirectly with an antiglobulin conjugate (procedure 2), or a protein A enzyme conjugate (procedure 3) (Mowat and Dawson, 1987). This simple approach is unsuitable for detecting viruses such as luteoviruses and geminiviruses that are present at low concentration in infected tissue. The method can be used when purified preparations of viral antigen are available, although it should be remembered that different viruses may have widely different capacities for sticking to polystyrene or polyvinyl plates. In addition, some viruses may be degraded in the presence of pH 9.6 carbonate buffer commonly used during the coating procedure; the viral subunits produced as a result of such degradation may become preferentially adsorbed to the solid phase, which could prevent any effective adsorption of virus particles. If a viral antiserum reacting both with the intact virions and dissociated subunits is used, positive detection will still occur, although a quantitative titration of the different viral antigens may become difficult. If monoclonal antibodies specific for either the polymerized or dissociated form of viral protein are used in the assay, results may become erratic (Van Regenmortel, 1986). Furthermore, it is now generally recognized that proteins become at least partly denatured or distorted when they are adsorbed to a layer of plastic during a solid-phase assay (Soderquist and Walton, 1980; Kennel, 1982; Friguet *et al.*, 1984; Altschuh *et al.*, 1985; McCullough *et al.*, 1985). The increasing use of monoclonal antibodies which are often specific for a conformation of the protein antigen has shown that this phenomenon is very common (Al Moudallal *et al.*, 1985; Vaidya *et al.*, 1985).

The most commonly used indirect EIA methods are the procedures 5 and 6 in Table I. Viral antiserum must be obtained from two different animal species. The most convenient animals for this purpose are rabbits, chickens, mice, rats, and goats. Since chicken globulins do not cross-react with mammalian Ig (Leslie and Clem, 1969), they are particularly useful, as they will not be recognized, for instance, by a goat antirabbit globulin conjugate (procedure 5, Table I). Another advantage of using chicken antibody is that the antivirus Ig can be obtained easily from the eggs of hens immunized with the virus (Polson *et al.*, 190; Van Regenmortel, 1982). Laying hens are convenient animals to house, and as much as 100 mg Ig can be extracted from one egg yolk (Gardner and Kaye, 1982). Although some investigators consider the need for a second virus antiserum obtained from another animals species a major inconvenience of the indirect immunoassay approach, this requirement can in fact be easily met. In the case of most plant viruses, there is no difficulty in obtaining quantities of the order of 1–10 mg purified virus, sufficient to immunize several rabbits, as well as a few chickens or even a goat.

The need for employing antibodies from two animal species can be circum-

vented by using $F(ab')_2$ fragments of antivirus Ig for coating microtitre plates (Barbara and Clark, 1982). The virus is trapped by the $F(ab')_2$ fragments and can be detected with the corresponding whole immunoglobulin followed by a protein A conjugate or by an anti-Fc fragment conjugate (procedures 6 and 7, Table I). It is a matter of opinion whether the need to prepare $F(ab')_2$ fragments is a simpler requirement than the preparation of antibodies in two animal species. However, the method has been used successfully to detect relationships among carlaviruses that were overlooked when direct EIA was used (Adams and Barbara, 1982). The use of the C1q component of complement instead of $F(ab')_2$ fragments for circumventing the need for two different virus antisera (Torrance, 1980) has not become popular, probably because C1q is not readily available commercially.

Recently, another EIA method using protein A was also been shown to overcome the excessive specificity of direct EIA procedures (Edwards and Cooper, 1985). Using this method, the wells of the microtiter plate are first coated with 1 μg/ml protein A, before incubation with the virus antiserum (procedure 13, Table I). The layer of protein A traps the antibody molecules by the Fab end. After incubation with antigen, the trapped virus is detected by another layer of antibodies (from the same antivirus serum), in turn detected by labeled protein A. This method has been shown to work satisfactorily with crude virus antiserum (diluted 1 : 1000) and eliminates the need to prepare Ig.

2.3. Use of Monoclonal Antibodies

Monoclonal antibodies (MAbs) have been produced against about 40 different plant viruses (Van Regenmortel, 1986). These reagents are especially interesting in the case of viruses, such as the luteoviruses or geminiviruses, which are present in only very low concentration in infected plants and which can be purified only with difficulty. Compared with conventional polyclonal antisera, the advantages of MAbs are (1) production of homogeneous and biochemically defined reagents in practically unlimited quantity, (2) increased specificity, and (3) easier immunization requirements, as mice can be immunized with virus preparations that are only partially purified.

The immunoassay most commonly used with MAb is some form of EIA. During the production of hybridomas, the clones secreting the desired antibody are mostly selected by an EIA that is adjusted to ensure maximum sensitivity of antibody detection. In a competitive study of several EIA procedures used for screening hybridoma cell lines, it was found that multilayered sandwich procedures (e.g., procedure 11, Table I) were usually the most sensitive (Al Moudallal *et al.*, 1984). However, it should be emphasized that the quality of the reagents used in such comparisons is of crucial importance, since the alleged superiority of a certain procedure may simply reflect the use of a reagent of exceptionally high titre.

The type of EIA used in the selection of the hybridoma can determine the properties of the MAb obtained. This is illustrated in Tables II and III in the case of MAbs raised against tobacco mosaic virus (TMV) and TMV coat protein, respectively (Altschuh *et al.*, 1985; Al Moudallal *et al.*, 1985). When clones were selected by an EIA procedure with antibody-coated plates (procedures 8 and 11, Table I), most MAb were obtained that recognized the native structure of the antigen. When the selection was done by means of antigen-coated plates (procedure 9), a large number of MAb were found to be specific for dissociated viral subunits or for a partly denatured form of the viral protein. Different EIA procedures will thus preferentially select MAb that recognize epitopes specific for the quaternary structure of the viral protein, i.e., the so-called neotopes (Van Regenmortel, 1966, 1982) or alternatively MAb that recognize epitopes specific only for the dissociated viral protein, i.e., the so-called cryptotopes (Dekker *et al.*, 1987). In addition, MAb can also be obtained that recognize epitopes common to both virions and subunits, i.e., metatopes (Table III).

2.4. Biotin Labeling

When the aim is to develop a double antibody sandwich assay using only MAb, the format of procedure 12 should be used (Diaco *et al.*, 1985). Biotin labeling of antibodies is made particularly simple using N-hydroxysuccinimido-biotin (Sigma, St. Louis). This activated biotin (0.2 mg) in dimethylformamide is simply incubated with the globulin (1 mg) in phosphate-buffered saline for 4 hr at 25°C (Zrein *et al.*, 1986). Labeling of the antibodies with biotin instead of with enzymes is not only advantageous in the case of MAb (Diaco *et al.*, 1985); biotin is a small molecule (244 M_r) that can be covalently linked to antibody

Table II
Reactivity of 18 Anti-TMV Monoclonal Antibodies with Virion and Subunit Preparations in Two EIA Procedures[a,b]

Monoclonal antibodies	EIA procedure 1[c]		EIA procedure 2[d]	
	Virions	Subunits	Virions	Subunits
Group 1 (8 clones)	+	−	−	−
Group 2 (5 clones)	+	+	−	−
Group 3 (5 clones)	+	+	+	+

[a]Data from Altschuh *et al.* (1985).
[b]For explanation of symbols, see Table I.
[c]EIA procedure 1 uses antibody-coated plates and consists of the following steps: Ab^R, Ag, MAb, $antiM^R$-E.
[d]EIA procedure 2 uses antigen-coated plates and consists of the following steps: Ag, MAb, $antiM^G$-E.

Table III
Reactivity of 30 Anti-TMVP Monoclonal Antibodies with Virion and Subunit Preparations in Two EIA Procedures[a]

Monoclonal antibodies	EIA procedure 1[b]		EIA procedure 2[c]	
	Virions	Subunits	Virions	Subunits
Group 1 (3 antineotope MAb)	+	−	−	−
Group 2 (5 anticryptotope MAb)	−	+	+	+
Group 3 (22 antimetatope MAb)	+	+	−	−

[a]Data from Al Moudallal *et al.* (1985).
[b]EIA procedure 1: Ab^G, Ag, MAb, antiM^G-E.
[c]EIA procedure 2: Ag, MAb, antiM^G-E.

without affecting its antigen-binding capacity (Guesdon *et al.*, 1979; Kendall *et al.*, 1983). The attachment of several biotin molecules to viral antibody is less detrimental to its capacity to recognize a broad range of serologically related viruses than is conjugation to an enzyme. In a recent study using biotinylated anti-TMV antibodies, Zrein *et al.* (1986) showed that tobamoviruses differing serologically from TMV by SDI values of ≤ 4.0 could be readily detected. By contrast, when enzyme-labeled viral antibodies were used, tobamoviruses differing from TMV by SDI values of only 2.0 could not be detected (Van Regenmortel and Burckard, 1980). One of the major drawbacks of DAS–EIA, i.e., excessive strain specificity, is thus overcome when the viral antibodies are labelled with biotin instead of with enzyme. Another advantage of using the biotin–avidin system is that the test is rendered more sensitive (Adler-Storthz *et al.*, 1983; Kendall *et al.*, 1983; Yolken *et al.*, 1983). A disadvantage of the biotin system is that it requires an additional incubation step with avidin. However, this can be avoided by incubating the biotinylated antibody together with the enzyme-labeled avidin; the resulting slight decrease in sensitivity can be compensated for by using the avidin conjugate at a higher concentration.

It has been claimed (Hill *et al.*, 1984; Diaco *et al.*, 1986) that when viruses are tested by DAS–EIA, it is necessary to use coating antibodies and labeled antibodies specific for different epitopes of the virus. However, since virions always possess a large number of identical epitopes (one per subunit), enough accessible epitopes remain free after trapping of the virus on the solid phase to permit subsequent binding of biotin-labeled antibodies of the same specificity (Dekker *et al.*, 1987). It should be remembered that different MAb may lose some of their binding activity after adsorption to plastic or after labelling; as a result, a test using the same antibody in both positions may appear inferior if the

stability of the antibody is affected in one or the other position (Torrance and Pead, 1986).

2.5. Specific Applications of EIA in Plant Virology

2.5.1. Diagnosis

Enzyme immunoassay has replaced most of the older serological techniques used for the diagnosis of plant viruses (Van Regenmortel, 1982), mainly because it is much more convenient for large-scale testing. The major limitation of analyzing large numbers of samples by EIA resides in the preparation of extracts of infected plant material. Tissue grinding can be done in mortars or with electrically driven devices (Banttari, 1980; McLaughlin *et al.*, 1984). Alternatively, leaf homogenates can be replaced by discs of leaf tissue cut out with a paper punch (Marco and Cohen, 1979; Romaine *et al.*, 1981).

The use of crude plant extracts in EIA may lead to a high level of non-specific reactions. These can be diminished by diluting the extract 10- or 100-fold or by the addition of substances such as 1–2% polyvinylpyrrolidone (Clark and Adams, 1977), mercaptoethanol, or dithiothreitol (Tamada and Harrison, 1980), diethyldithiocarbamate (McLaughlin *et al.*, 1984), 1 M urea (Gugerli, 1979), and nicotine (Walter *et al.*, 1984). There are no rules about which substances will be found advantageous or deleterious in a particular instance, and the best extraction medium must be selected empirically (Huss *et al.*, 1986). Instead of performing EIA in the usual microtiter plates, other solid-phase materials such as polystyrene and frosted glass beads have also been used for detecting plant viruses (Felgner, 1978; Chen *et al.*, 1982; Polson *et al.*, 1985).

Enzyme immunoassay procedures are widely used for large-scale investigations of the incidence of viral infections in various crops such as potatos (Gugerli, 1979; Tamada and Harrison, 1980), deciduous fruit (Barbara *et al.*, 1978; McMorran and Cameron, 1983), citrus (Lommel *et al.*, 1983; Stein *et al.*, 1979), vegetables (Jafarpour *et al.*, 1979; Marco and Cohen, 1979), grain (Lister *et al.*, 1981; Rochkow, 1979), and grapevines (Gonsalves, 1979; Walter *et al.*, 1984).

2.5.2. Virus Detection in Vectors

Because of their sensitivity, EIA procedures are particularly suitable for detecting plant viruses in their insect vectors (Gera *et al.*, 1978; Clarke *et al.*, 1980; Tamada and Harrison, 1981; Carlebach *et al.*, 1982; Ghabrial and Schultz, 1983; Caciagli *et al.*, 1985). In this connection, increased sensitivity of antigen detection by the use of a fluorogenic substrate (Torrance and Jones, 1982) and the amplified EIA technique (Torrance, 1987) has been beneficial. The ability of

detecting virus in single viruliferous insects has opened the way to new epidemiological investigations.

2.5.3. Quantitative Analysis of Antigenic Relationships

Until recently, there have been few attempts to use EIA for analyzing the antigenic relationships between plant viruses (Rybicki and Von Wechmar, 1981; Powell and Derr, 1983). Because of their sensitivity, EIA procedures will tend to bring out any reactions with contaminating antigens; this problem can often be circumvented using relatively high dilutions of the reagents. In all types of solid-phase immunoassays, it is important to avoid the use of too highly concentrated reagents, as this tends to emphasize nonspecific interactions (Altschuh and Van Regenmortel, 1983; Muller *et al.*, 1985). Electrostatic interactions between Ig and viral proteins can be prevented by the addition of heparin to incubation buffers (Dietzgen and Francki, 1987).

The degree of antigenic relatedness between two plant viruses is commonly expressed by the serological differentiation index (SDI), which corresponds to the average number of twofold dilution steps separating homologous from heterologous precipitation titers (Van Regenmortel and Von Wechmar, 1970). Unfortunately, SDI values calculated from different antisera to the same antigen can vary considerably, and a reliable estimate of the degree of cross-reactivity between two viruses can only be obtained by studying many bleedings from different immunized animals (Van Regenmortel, 1982). Jaegle and Van Regenmortel (1985) showed that reliable SDI values, corresponding to those measured in precipitation tests, could be obtained by indirect EIA measurements. The economy of reagents obtainable in EIA makes it easier to examine a large number of different antisera; furthermore, since EIA titers are usually much higher than precipitation titers (of the order of 10^{-6} instead of 10^{-3}), EIA can detect much more distant serological relationships than can the precipitin test (Hoffman and Van Regenmortel, 1984). For instance, a distant relationship between turnip crinkle virus and several tombusviruses (SDI = 10–13) that remained undetected by precipitin tests was shown by indirect EIA (Jaeglé and Van Regenmortel, 1985).

An indirect EIA method suitable for calculating relative titers of plant virus antisera and for deriving SDI values was recently described (Clark and Barbara, 1987). Data from the proportional response region of each experimental curve were converted to logarithmic values and subjected to regression analysis to obtain relative titers. The method permitted closely related viruses to be discriminated in a reproducible manner and demonstrated the potential value of EIA for quantitative antigenic analysis.

2.5.4. Analysis of Antigenic Structure

The advent of EIA has made it possible to analyze the antigenic structure of plant viruses by fairly simple procedures. By means of EIA inhibition tests using peptide fragments (Altschuh and Van Regenmortel, 1982), the location of continuous epitopes in viral proteins can be easily determined (Quesniaux *et al.*, 1983). Synthetic peptides coupled to protein carriers have also been used in EIA to identify the epitopes of plant virus proteins (Al Moudallal *et al.*, 1985). When used in conjunction with MAb, various formats of EIA represent the most powerful methodology available for elucidating the antigen structure of plant viruses (Al Moudallal *et al.*, 1982, 1985; Briand *et al.*, 1982; Altschuh *et al.*, 1985; Doughtery *et al.*, 1985; Tremaine *et al.*, 1958; Koenig and Torrance, 1986).

3. CONCLUSIONS

The introduction of the EIA technique has been exceedingly important for the diagnosis of viral diseases, and its use in detecting molecules immobilized on a solid phase has revolutionized both clinical and research laboratories. Early methods were adopted because the tests were simple, inexpensive, fast, and sensitive. However, with the advent of highly specific reagents, such as monoclonal antibodies, the specificity of the methods has increased considerably. Moreover, techniques have been developed for the detailed study of the immunopathogenesis of viral infections.

The introduction of class-capture EIA has permitted a much better follow-up of management of the IgM and IgG immune responses than had been possible with classic methods and shown many erroneous assumptions about the relation between primary infection and the IgM response, particularly for viruses that lack antigenic uniformity (Meurman, 1983), such as enteroviruses, adenoviruses, parainfluenzaviruses, and togaviruses. Class-capture assays are also increasingly used for studying the response of the IgG subclasses of antibodies. Assays of this type indicate that antibodies often occur in one subclass after the primary infection but in another after a recurrent infection.

The trend toward developing highly specific reagents and innovative tests can be expected to continue and should lead to a better comprehension of the process of infection and of the body's defense systems. In addition, an increasing number of commercial EIA kits are available for the detection of infectious agents or their antibodies. The assays have become more rapid and simpler to perform.

Advances in recombinant DNA technology have permitted the use of nucleic acid probes in the diagnosis of human viral diseases. However, wide

application of these probes will occur only if enzymes or other nonradioactive detectors are used to determine whether the probes have hybridized to the specimen nucleic acid. This methodology is suitable both for quantitation and localization purposes and is a valuable adjunct to the enzyme immunoassay.

The examples described illustrate the application of EIA methods, or DNA probes, to the study of viral diseases that were previously difficult to diagnose. A multitude of papers have been published in recent years with EIA in the titles, attesting to the popularity of the technique and to the difficulties of reviewing this topic comprehensively.

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