Reviews
Analyses

Bulletin of the World Health Organization, 64 (3): 465–479 (1986)

Enzyme immunoassays and related procedures in diagnostic medical virology

EDOUARD KURSTAK,[1] PETER TIJSSEN,[2] CHRISTINE KURSTAK,[3] & RICHARD MORISSET[4]

This review article describes several applications of the widely used enzyme immunoassay (EIA) procedure. EIA methods have been adapted to solve problems in diagnostic virology where sensitivity, specificity, or practicability is required. Concurrent developments in hybridoma and conjugation methods have increased significantly the use of these assays. A general overview of EIA methods is given together with typical examples of their use in diagnostic medical virology; attention is drawn to possible pitfalls. Recent advances in recombinant DNA technology have made it possible to produce highly specific nucleic acid probes that have a sensitivity approximately 100 times greater than that of EIA. Some applications of these probes are described. Although the non-labelled nucleic acid probes for use in the field are not as refined as non-labelled immunoassays, their range of applications is expected to expand rapidly in the near future.

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 towards 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. Moreover, serological approaches are important for distinguishing between serotypes or isolates. Developments in the design of enzyme immunoassays (EIA) and the production of monoclonal antibodies have enabled techniques to be developed that have a specificity and sensitivity approaching or surpassing that required for clinical applications but which do not suffer from the serious drawbacks of classical methods. In addition, enzyme-labelled probes for the detection of specific sequences of viral nucleic acid have permitted the development of even more sophisticated techniques that are approximately 100 times more sensitive than classical enzyme immunoassays (typically, 1–5 pg of substance is detected). These new methods take advantage of recent advances in molecular biology, particularly in DNA mapping, cloning, and sequencing.

[1] Professor and Director, Comparative Virology Research Group, Faculty of Medicine, University of Montreal, Montreal, P.Q., H3C 3J7, Canada. Address to which requests for reprints should be sent.

[2] Research Assistant, Comparative Virology Research Group, Faculty of Medicine, University of Montreal, Montreal, Canada.

[3] Head, Laboratory of Virology, University of Montreal, Hospital Hôtel-Dieu de Montreal.

[4] Chief, Service of Microbiology, University of Montreal, Hospital Hôtel-Dieu de Montreal.

USE AND MISUSE OF EIA IN DIAGNOSTIC VIROLOGY

Virtually all enzyme immunoassays used in virology are carried out in the solid phase. The variety of

assays may appear confusing and may be responsible for their frequent inadequate use. Disregard of the underlying principles governing these assays has frequently led to disappointing results, despite the method's great potential.

Enzyme immunoassays have recently been evaluated theoretically (*69*, *70*, *132*) and classified into two fundamentally different groups: activity amplification (AA) and activity modulation (AM) assays. AA assays are extremely sensitive and employ a large excess of labelled detector molecules. In contrast, in AM assays emphasis is placed on specificity rather than sensitivity, by weighting the affinity constants of the various antibody–antigen systems present in the assay. The requirements for higher specificity or higher sensitivity depend on the prevalence of the disease being studied, since their interrelationship follows Bayes' theorem. Unfortunately, however, these factors are seldom taken into account in the development and improvement of enzyme immunoassays. Despite the frequent requirement for greater specificity, investigators opt almost without exception for the inappropriate enzyme-linked immunosorbent assay (ELISA) technique. The suitability of ELISA methods in this respect will only increase, however, when detector molecules of greater intrinsic specificity have been developed. This is generally a difficult procedure and improvements can be made much more readily by modifying the EIA system.

USE OF NUCLEIC ACID PROBES IN ENZYMATIC DETECTION OF SPECIFIC VIRAL GENOME SEQUENCES

Developments in recombinant DNA technology made possible the use of nucleic acid probes in diagnostic virology. Although most studies have used radioisotopes or fluorescent labels for the detection of *in situ* hybridization, enzymes offer very significant advantages.

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 and human papilloma virus. The specific viral DNA fragments can be further digested by restriction endonucleases and subcloned into plasmid vectors, thus enabling single- or double-stranded nucleic acid (RNA or DNA) viral diagnostic probes to be prepared. This dot-blot hybridization technique is considerably more sensitive than other methods and may account for its rapidly growing applications. An added advantage of the technique is that, in addition to free nucleic acid, integrated DNA can be detected (*119*).

Dot-blot hybridization is particularly useful in the study of herpesviruses. For example, using this technique the various types of human (alpha) herpesvirus could be distinguished at very low concentrations (*125*), although this has recently also been reported using monoclonal antibodies (*69*). The technique has also enabled cytomegalovirus to be detected directly in the urine of immunosuppressed patients (*20*) and the discrimination of types of this virus (*124*). Similarly, Epstein-Barr virus has been detected at a level of 0.1–0.5 genome equivalents per cell (*10*). Most probes used in this technique are radiolabelled with ^{32}P. It should be noted, however, that 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 similar way to enzyme immunoassays (*83*). The degree of stringency of the hybridization conditions determines the degree of complementarity in the duplex strands of DNA. Use of stringent conditions (about 25 °C below the melting temperature) allows only formation of well-matched duplexes (*54*). However, lower hybridization temperatures permit detection of related sequences. For example, under stringent conditions a probe for human papillomavirus type 1 (HPV1) detects only this virus, but at lower hybridization temperatures HPV4, HPV6, and HPV8 are also detected (*7*).

Nucleic acid probes are not only extremely important in diagnostic virology but also in viral histopathology and viral epidemiology. For example, Brigati et al. detected viral genomes (parvovirus, polyomavirus, herpesvirus, adenovirus, and retrovirus) in both cultured cells and paraffin-embedded tissue sections using biotin-labelled hybridization probes (*12*). The use of specific tracers also provides information about the possible origin and transmission routes of infectious agents in epidemics (*43*, *120*).

ENZYME LABELLING OF NUCLEIC ACID PROBES

Advances in molecular biology have made it relatively simple to obtain parts of the viral genome that are distinct (e.g., in virulence) from related viruses, to clone or subclone these fragments in appropriate vectors, and to produce large amounts of these recombinant probes by transformation of bacteria (*87*). Moreover, this makes it possible to produce reagents for viruses, such as hepatitis virus B and human parvovirus, that cannot be grown *in vitro* (*21*).

Almost all nucleic acid probes are currently labelled with ^{32}P by the *in vitro* nick translation technique using (α-^{32}P)-dUTP in the presence of the

three other (cold) dUTPs. Probes labelled with ^{32}P have several disadvantages: they have a half-life of 2 weeks, require well-trained technicians, are in constant danger of accidental contamination, are costly, and pose disposal problems. In contrast, enzymes form stable reagents that have a half-life of at least 1 year; furthermore, the tests are then much less costly to perform and can be used universally without serious health risks. The sensitivity of enzyme-labelled probes is comparable to that of radio-labelled probes. To prepare enzyme-labelled DNA probes caproylamidobiotinyl-*N*-hydroxysuccinimide esters are conjugated to 5-(3-amino)allyldeoxyuridine triphosphate (*83*), and the conjugates are then introduced into DNA by nick translation. DNA probes that react with streptavidin-labelled enzyme represent the state of the art for identification of viruses, and several DNA-probe diagnostic kits should become commercially available within the next year. Kits for the in-house preparation of DNA probes based on enzyme detection are already commercially available.[a]

Adaption of the dot-hybridization test for routine diagnosis is relatively straightforward. For example, a few microlitres of diluted serum is applied to a nitrocellulose membrane, denatured *in situ* (*89*), filter-hybridized by standard methods, and the enzyme detected using procedures similar to those employed in enzyme immunohistochemistry (*71*, *74*, *78*, *79*). Serological enzyme immunoassay and genomic (enzyme-linked DNA probes) tests are complementary, each providing unique information: DNA probes are suitable for the direct detection of the virus or its immune complexes, for which enzyme immunoassays are frequently least sensitive, whereas enzyme immunoassays are the method of choice for detecting viral antibodies.

Like immunohistochemical techniques, biotinylated DNA probes are suitable for the detection and localization of specific viral sequences in infected cells (*12*). For this purpose, the use of streptavidin-labelled instead of avidin-labelled enzymes is recommended, since avidin may react nonspecifically with nucleic acids.

SEROLOGICAL ASPECTS OF IMMUNOASSAYS

1. *IgM determinations: usefulness and pitfalls*

The humoral immune response to viral infections generally involves production of IgG antibodies, but with individual variation of the effect of these antibodies on the course and convalescence of the infection (*24*). However, in the initial response to a primary viral infection transient IgM antibodies are often produced, and it has been suggested (*118*) that determination of the ratio of IgM:IgG may be a valuable tool in identifying such infections. The detection of antiviral IgM antibodies is, therefore, widely used in diagnosis (*92*). This approach is, however, only valid provided the IgM:IgG ratios of the antibodies produced by the humoral response do not vary between individuals and that the levels of both IgM and IgG can be measured with sufficient reliability and sensitivity.

2. *Humoral immune response to viral diseases*

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 in young children, immunocompromised individuals, local infections (e.g., respiratory), and reinfections or reactivation of latent infections. Meurman noted that the efficacy of IgM tests for confirmed respiratory infections of young children by parainfluenza virus (type 1-3), respiratory syncytial virus, and adenovirus was positive in only 25-63% of cases (median age range of patients, 11-18 months) (*92*). Moreover, Welliver et al. observed that the IgM response to respiratory syncytial virus infections of 1-3-months-old children was much weaker that that of children aged 3-12 months (*141*). These virus infections are often too localized to generate a systemic immune response (*25*, *116*, *134*). Furthermore, IgM responses are absent in about 25% of influenza virus A or B infections (*40*), and similar phenomena have been observed with echovirus, coxsackievirus, and rotavirus infections (*90*, *112*).

Though the absence of an IgM response after rubella infections has been regarded as indicative of reinfection (*9*), an IgM response has been recorded in about 20% of vaccinated subjects reinfected with live rubella virus (*46*). The IgM antibody response to reactivation of latent infections of alphaherpesvirus 1 and 2 is rare, but frequent (70-80%) in cases of alphaherpesvirus 3 (*92*). Reactivation of cytomegalovirus sometimes results in high IgM responses (*17*), although low values have also been reported (*59*, *135*).

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 (*13*, *140*); for example, rubella virus IgM response of up to 4 years has been observed (*126*). After renal transplantation in immunosuppressed patients, cytomegalovirus, and

[a] From Bethesda Research Laboratories, Inc., Gaithersburg, MA, USA and ENZO Biochemicals, New York, NY, USA.

BK papovirus are often reactivated followed by a prolonged IgM response (*17*, *34*). Patients with chronic viral hepatitis B or "healthy" carriers have also frequently a prolonged IgM antibody response (*115*).

It should be noted, however that the detection of these IgM responses may simply arise because of the high sensitivity of the assays compared to less sensitive tests. The usefulness 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 (*92*). However, viruses for which several strains or serotypes exist lead to complications in the IgM response (e.g., absence of IgM response, false positive reactions). The increased specificity and sensitivity of enzyme immunoassays is having a profound impact on the determination of specific IgM antibodies for these heterogeneous groups of viruses such as enteroviruses, togaviruses, parainfluenza virus, and adenoviruses.

In recent years, enzyme immunoassays 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. IgG_1 is the major subclass, and antibodies to viruses are usually found in this subclass, though subclass IgG_3 antibodies are also frequently found (*84*, *99*, *129*). IgG_2, the second subclass, as a whole appears mainly to contain antibodies to polysaccharides, e.g., lipopolysaccharides of bacteria. Subclass IgG_3, which does not react with protein A rheumatoid factors, has a rapid turnover and is the most active subclass in activating the C1 component of complement; it is frequently associated with recurrent illnesses (*38*). Subclass IgG_4 antibodies have been detected after herpesviral infections. Interestingly, IgG_4 responses to herpes simplex virus are common, but for cytomegalovirus and alphaherpesvirus 3 (varicella-zoster virus) a familial relationship has been observed (*38*, *137*), indicating that IgG_4 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.

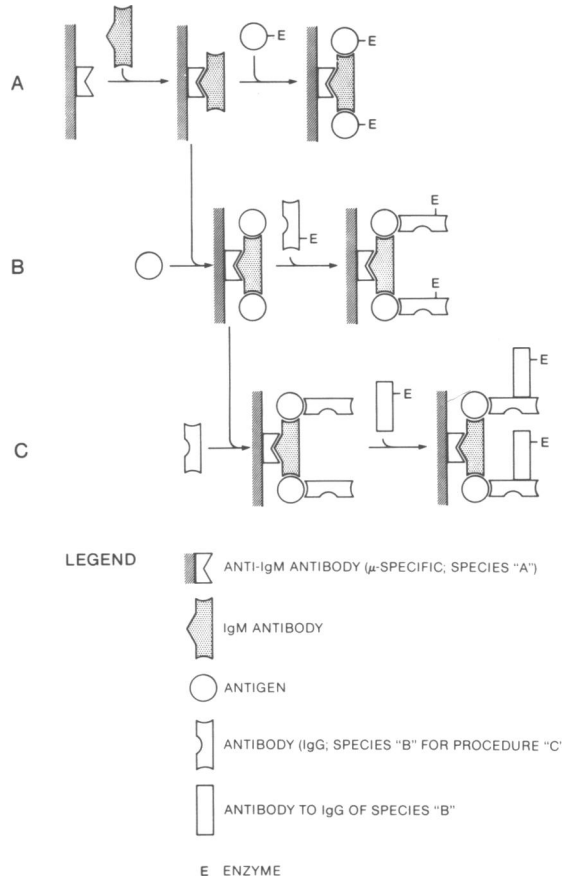

Fig. 1. Principles of class-capture assays. The anti-immunoglobulin class antibody (e.g., IgM) is adsorbed on the solid phase and will trap molecules of that class. If antibodies are contained within this immunoglobulin class, antigen added subsequently will also be immobilized (A). This antigen can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

3. *The detection of IgM antibodies*

Three major problems may be encountered in the detection of IgM antibodies: competition between IgM antibodies and antibodies of other classes for the antigen; the frequently lower affinity of IgM antibodies relative to IgG antibodies, resulting in distorted test results; and differentiation between the immunoglobulin classes.

Early methods, which are still widely used, to distinguish IgM from IgG titres were based on the physical separation of IgM from IgG by sucrose-density gradient fractionation, gel filtration, or affinity- or ion-exchange chromatography. Alternatively, IgM molecules can be selectively dissociated by thiols to produce subunits with lower activity. However, to produce significantly different titres for untreated and treated serum at least 75% of the total antibodies should be of the IgM class; only a few viruses cause such marked IgM immune responses, e.g., tick-borne encephalitis (*68*). Nevertheless, this method, in conjunction with physical separation methods, can be used. For example, rheumatoid

factors or heat inactivation may enable IgG antibodies to be recovered from IgM fractions (*39*, *107*). Treatment with thiols is also necessary if protein A is used for the removal of IgG, since in this case not all subclasses are removed to the same extent. Human IgG_3 and a large fraction of IgA are not absorbed by protein A (*66*), and this would lead to false positive results.

Duermeyer & van der Veen (*29*) reported class-capture enzyme immunoassays (Fig. 1) that avoid the problems caused by competition of IgG and IgM antibodies for the same antigen. These assays are mainly used for hepatitis viruses, rubella viruses, flaviviruses and herpesviruses. A possible drawback is that the sensitivity of, for example, an IgM-capture assay will be influenced by the fraction of specific IgM antibodies in the total IgM pool (*48*) and by the presence of rheumatoid factor, where nonspecific interactions remain possible as in other assays (Fig. 2).

Nonspecific adsorption of IgM seems to be higher than that of IgG (*92*) because of the law of mass action. Rheumatoid factors have a steep dose–response curve (*95*) and dilution is one way of decreasing their interference. Pregnancy is quite often accompanied by the activation of rheumatoid factors, and current methods to decrease their interference with immunoassays involve the use of IgG aggregated by heat (*78*) or glutaraldehyde (*65*), latex particles covered with IgG (*138*), or excess normal IgG (*96*); however, 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 enzyme immunoassays are shown in Fig. 3.

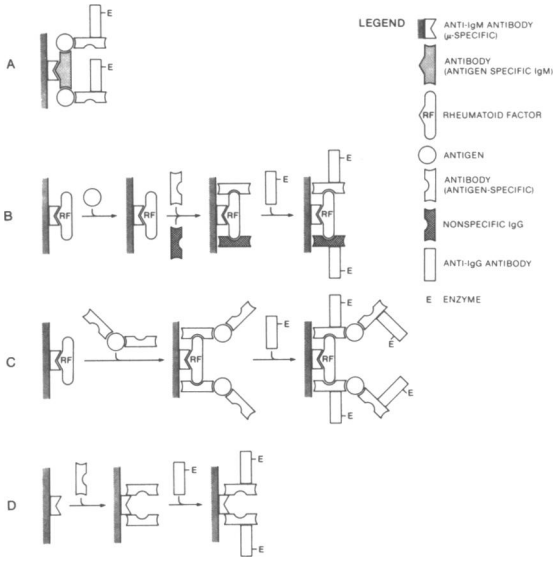

Fig. 2. Class-capture assay (A) (performed as shown in Fig. 1) and possible causes of nonspecific interactions (B, C, D). Anti-IgM assays may cause the nonspecific adsorption of rheumatoid factors (RF) which adsorb IgG without the presence of antigen (B) or immune complexes (C). Moreover, nonspecific protein–protein interactions (D) may cause background staining.

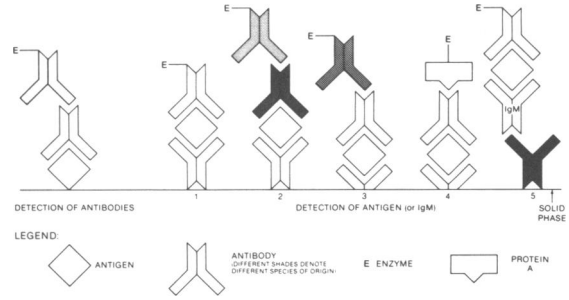

Fig. 3. Frequently used enzyme immunoassays for the detection of antibodies, antigen (1–4), or antibodies belonging to specific immunoglobulin classes (5). The molecules to be detected are always found in the second layer.

SOME APPLICATIONS IN MEDICAL VIROLOGY

1. *Viral hepatitis*

Viral hepatitis B is one of the most persistent viral infections of man with over 200 million people chronically infected. Although viral hepatitis A is never associated with chronic liver infection, it is readily transmitted orally and is a health problem in endemic regions. Following the introduction of suitable screening methods, hepatitis virus non-A, non-B has been identified as the agent of the most important post-transfusion hepatitis. The hepatitis delta agent is distinct from hepatitis B virus (HBV) but requires the presence of the latter to replicate.

Wolters et al. (*145*, *146*) developed an ELISA test for hepatitis virus B surface antigen (HBsAg) and this is now marketed commercially under the name Hepanostika HBsAg.[b] Similarly, another laboratory has offered an ELISA test for the same antigen.[c] In a recent analysis, Wolters et al. (*145*) found very few false positives (depending on the cut-off value about

[b] From Organon Teknika, Turnhout, Belgium.

[c] From Abbott Laboratories, North Chicago, IL, USA.

1%) both in rheumatoid factor-positive and negative sera. Although Gadkari et al. (*37*) reported excellent results with peroxidase conjugates prepared with 3-(2-pyridyldithio) propionate for the detection of HBsAg, we found the stability of these conjugates to be poor.

The presence of HBsAg in blood is frequently used as an indicator of HBV infection and concentrations may range from 0.001 to 50 μg/ml, although HBsAg is a poor marker for infections since 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 viral hepatitis B infections (*31*, *47*, *131*).

The results of *in situ* hybridization of HBV DNA in the cytoplasm of liver cells (*15*) and the presence of mainly "-" strand viral DNA in the cytoplasm of hepatocytes are in accord with the hypothesis that hepatitis virus B replicates via an RNA intermediate.

Detection of weak anti-hepatitis B core (HBc) IgM response in HBsAg-positive patients cannot always be considered to be a definitive marker of recent infection. However, quantitation of HBc IgM in HBsAg carriers using class-capture ELISA increases the clinical usefulness of the test and permits differentiation between acute HBV infections and jaundice due to other causes (*67*). Anti-HBc IgM class-capture ELISA tests are important since HBsAg carriers generally exhibit very high total antibody titres for anti-HBc.

Papaevangelou et al. (*105*) showed that the diagnostic value of anti-HBc IgM in areas where the prevalence of hepatitis virus B is high is important because complex diagnostic situations arising from other forms of acute liver injury may superimpose on a patient with chronic HBV liver disease.

The delta agent can be detected by the immunoperoxidase method (*111*), and by ELISA for determining total anti-delta antibodies (*121*), or IgM using class-capture assay (*122*). The potential importance of delta agent has been stressed by Smedile et al., who found that the incidence of delta markers was 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 ELISA test for delta agent with radioimmunoassay (*121*).

2. *Viral gastroenteritis*

Viral gastroenteritis is common worldwide, its prevalence being second probably only to that of respiratory illnesses (*32*). Its clinical features vary, and, after a rapid onset, include nausea, vomiting, abdominal cramps, headache, anorexia, myalgia, and malaise. The malabsorption triggered by gastroenteritis viruses may increase morbidity in poorly nourished populations. Other cytopathic enteroviruses, e.g., poliovirus and coxsackievirus, do not normally induce this syndrome.

Rotaviruses infect the gastrointestinal 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 (*58*). Cultivation of human rotavirus in the laboratory is not satisfactory for diagnostic purposes (low isolation rates and the need for several passages). Solid-phase enzyme immunoassays have proved useful in this respect (*147*); however, nonspecific reactions frequently occurred in early studies (*64*). Nevertheless, various modifications, such as the use of chelating agents, protease inhibitors, pH-neutralization, and the inclusion of normal sera, greatly improved the reliability of these diagnostic tests (*3*, *44*, *50*, *53*). A commercial kit, Rotazyme,[d] uses simian rotavirus (SA11), which shares a common group antigen with the human rotaviruses, and contains an anti-simian rotavirus serum; however, it is unreliable for neonates (*64*). Both the sensitivity and specificity of the assay can be increased by addition of a 0.25 mol/l solution of ethylenediaminetetraacetic acid (*23*). A method using monoclonal antibody to the common rotavirus antigen is superior in both sensitivity and specificity to the Rotazyme test (*26*). Recently a new type of rotavirus ("pararotavirus") has been discovered that lacks the group-specific antigen, but its medical importance is not yet clear.

Electron microscopy is still frequently used to detect rotaviruses, while the immunoperoxidase method is used to detect their antigens (Fig. 4A). On the other hand, the sensitivity of dot-blot hybridization is such that the detection of only 8 pg of viral RNA has been reported; this method is therefore 10–100 times more sensitive than ELISA (*33*) and also permits discrimination of various viral strains (*128*). Recently an enzyme-linked cell immunoassay for detection and titration of rotavirus antigen has been developed. In this technique the advantages of the cell culture system for virus isolation are combined with those of enzyme immunodetection and spectrophotometry (*42*).

An equally important gastroenteritis virus is the Norwalk virus. Though it was originally assumed to be a parvovirus, the discovery that it contains only one protein of relative molecular mass 66 000 (*41*) makes it more probable that it is a calicivirus. The low shedding rate of the Norwalk virus has so far

[d] See footnote *c*, page 469.

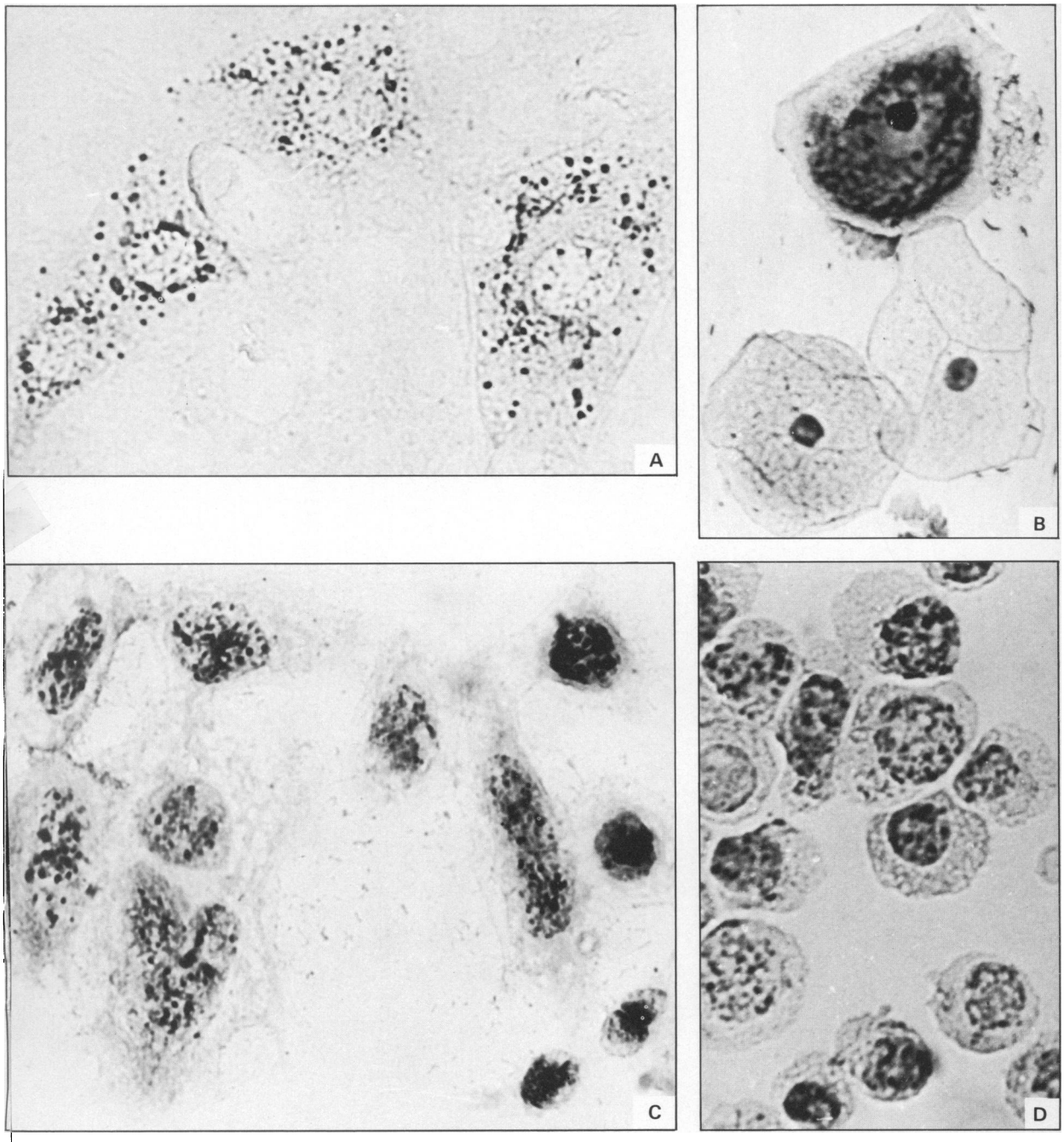

Fig. 4. (A) Rotavirus antigens localized in the cytoplasm of BS-C-1-infected cells by immunoperoxidase staining. (B) Cyto-smears of cervical scraping from a patient infected with genital herpesvirus showing immunoperoxidase staining of alphaherpesvirus 2 antigens. (C) Alphaherpesvirus 2 intranuclear antigens detected by immunoperoxidase staining in human Hep 2 cells. (D) Anti-complement immunoperoxidase staining of Epstein-Barr virus nuclear antigen in human lymphocytes.

hampered its characterization and the preparation of a hyperimmune serum. Infantile gastroenteritis is often caused by one of the new serotypes of adenoviruses (*11*, *27*).

3. *Herpesvirus infections*

Numerous serological tests and other procedures have been developed for the specific detection of antibodies to alphaherpesvirus 1 and 2 in human sera, e.g., neutralization kinetics, microneutralization, indirect haemagglutination, immune lysis of infected cells, immunofluorescence, immunoperoxidase assay, solid-phase radioimmunoassay, immunoelectrophoresis, and polyacrylamide gel electrophoresis after immunoprecipitation (*45*, *77*, *79*, *80*). Microneutralization is most frequently used in seroepidemiological studies (*108*, *110*), but ELISA methods are faster and easier to perform (*22*, *139*). Hampar et al. have developed a modified ELISA test (*45*) 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%.

It has been suggested that IgE contributes to the pathogenesis of herpesvirus infections (*55*); 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. (*136*) devised a sensitive, direct ELISA test based on the antibody-capture principle (sequence: affinity-purified, anti-human IgE-coated microtitration plates; sample with IgE antibodies; peroxidase-labelled viral antigens). This method may become an important tool for investigating the role played by the IgE response in immunological defence and viral pathogenesis and for testing its significance.

Cytomegaloviral infections are serious if recurrent, particularly in immunocompromised patients (*142*). McKeating et al. postulated that the presence of β_2-microglobulin in samples of fresh urine prevents detection of cytomegalovirus by electron-capture ELISA by binding to the virus, masking its antigenic determinants (*149*). Recently, a competitive ELISA method was developed for detecting cytomegalovirus 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 ELISA (*150*). Ida et al. obtained evidence that the IgE response to herpesviral infection is important in patients with decreased numbers of T-suppressor cells because of immunosuppressive therapy (*55*). Very conclusive results were obtained with alphaherpesvirus 2 localized within 60 minutes in cells obtained by cervical scraping (Fig. 4B) or in cells infected *in vitro* (Fig. 4C), and for Epstein-Barr virus nuclear antigen (Fig. 4D) using direct immunoperoxidase or anticomplement immunoperoxidase techniques (*73*, *76*, *77*, *83*).

4. *Rubella*

Maternal rubella is transmitted to the fetus by way of viraemia, particularly during early pregnancy (*97*), 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, re-infection during pregnancy of previously vaccinated women, acute rubella infection or contact with infected individuals, and rubella immunity during pregnancy. The enhanced awareness of the problems associated with rubella infection is an increasing reason for termination of pregnancy (*30*).

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. Detection of rubella-specific IgM antibodies indicates recent infection by rubella. For the standard haemagglutination inhibition tests serum fractionation is necessary, but this is impractical for large-scale screening. Considerable research has gone into developing class-capture assays of the ELISA type for rubella (*56*, *138*). Physical separation of IgG from IgM antibodies need not be carried out and only 5 μl of sample suffices. In this respect, particularly the $F(ab')_2$ conjugates have proved reliable and sensitive probes.

The commercial kit, Rubazyme-M,[e] for the detection of rubella-specific IgM is not satisfactory; for example, Best et al. found that of 20 neonates who had confirmed intrauterine rubella 2 gave false-negative results (*4*, *5*), while Steece et al. reported that the predictive value for a negative result is only 36.4% using Rubazyme (*127*). On the other hand, the presence of rubella-specific IgM in patients with infectious mononucleosis may be caused by stimulation by Epstein-Barr virus of B lymphocytes specific for rubella or incorporation of cellular

[e] Abbott Diagnostics, North Chicago, IL, USA.

antigens into the envelope of the virus used as antigen (*101*).

5. *Measles*

Measles may be associated with complications such as prolonged diarrhoea, encephalitis, pneumonia, otitis media, and, sometimes, subacute sclerosing panencephalitis. The global case fatality rate in developing countries has been estimated to approach 2% (1.5 million deaths per year) (*88*).

ELISA is as sensitive as other serological techniques for detecting seroconversion to measles virus (*109*). A convenient and flexible ELISA method for detecting antibody to measles antigen was introduced by Rice et al. (*113*). 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 ELISA procedures. Boteler et al. have developed an ELISA test for IgG antibody to measles (*8*) that is sensitive, specific, and accurate (coefficient of variation: ± 5%). A coefficient of determination of 1.00 was obtained from three test centres, and the values obtained were linearly proportional to titres. Measles captive assays for IgM using monoclonal antibodies appear to be useful and produce little or no background values (*36*, *63*).

The paucity of anti-human IgM reagents of adequate specificity, potency, and consistency has hampered the development and application of viral IgM antibody assays. Forghani et al. produced monoclonal antibodies to human IgM (*35*) that were evaluated in capture-class assays for measles and rubella IgM antibodies (*36*). This approach avoided cross-reactivity with IgG and eliminated the need to include antibody-negative human sera in the diluents for the test reagents (*56*). False-positive reactivity caused by the presence of rheumatoid factors was virtually eliminated by using glutaraldehyde-aggregated IgG. Latex agglutination tests are poor indicators of rheumatoid factors and give high levels of false-positive reactivity.

O'Beurne et al. used the critical ratio (absorbance of convalescent sera: absorbance of acute sera) to detect an increase in the antibody titre (*104*). A four-fold increase in the complement fixation titre corresponded to a critical ratio of about 1.5. It should be noted, however, that such ratios depend heavily upon the serum dilution used.

In the USA the prevalence of measles has decreased dramatically since the introduction of measles immunization (*18*). Nevertheless, a diagnostic screening test to identify nonimmune individuals is important because of the severe complications that can occur after infection with measles virus (*100*). Haemagglutination inhibition tests have been widely used for this purpose but are inconvenient since sera should be pre-treated to remove the nonspecific inhibitors or agglutinin that would otherwise influence their reliability or specificity.

6. *Viral infection of the respiratory tract*

Most infections of the upper respiratory tract are caused by viruses, but bacteria may account for up to a third of such infections.

Conventional haemagglutinin and neuraminidase inhibition tests for influenza infections are relatively insensitive (*133*). However, ELISA methods are much more sensitive and permit the assay of antibodies in nasal washings. Murphy et al. developed an ELISA test for haemagglutinin instead of for the whole virus (*102*), whereas Khan et al. reported an ELISA test for neuraminidase-specific antibodies (*61*). A pitfall in the determination of titres for influenza viruses was pointed out by Madore et al. (*86*), who observed that dose–response curves could be displaced upwards, reflecting an increase in antibody activity, without a change in the end-point (steeper curves). Differentiation of influenza virus strains requires the use of their constituent proteins rather than of whole virus (*1*).

Parainfluenza viruses are common in respiratory illnesses of children. The main problems in standard methods for their determination are the rapid loss of infectivity of the viruses and the high cost of identifying them by cell culture techniques. Immune responses may take several weeks to be sufficiently intense for a positive identification. In contrast to aspiration with a mucus extractor (through the nostrils), use of nasopharyngeal washings gives low yields of parainfluenza antigen (*177*). Cross-reactivity between antibodies to parainfluenza virus types 1 and 3 as well as with antibodies to mumps virus can be significant (*57*). Live virus is not required for ELISA, and this is particularly important for the diagnosis of parainfluenza virus. For example, in this way Parkinson et al. were able to detect antigens to parainfluenza virus in cell cultures from which infectious virus could no longer be recovered (*106*).

Respiratory syncytial virus is also commonly diagnosed by cell-culture techniques. Here, enzyme immunoassays are, therefore, an important alternative method. The sensitivity of these assays is approximately 80% that of classical culture methods (*51*, *92–94*). The clinical sensitivity of ELISA is higher for respiratory syncytial viral infections of children under 6 months of age (*92–94*) and is superior to that of complement fixation (*52*). IgM responses are often absent. Respiratory syncytial virus antigens in naso-

pharyngeal secretions can be detected by enzyme immunoassay using goat and rabbit anti-respiratory syncytial virus as the capture and detector antibodies, respectively. Compared to cell-culture methods, the immunoassay has a sensitivity of 91.3% and a specificity of 96.8% (*151*). ELISA methods have also been used successfully to detect coronaviruses (*62, 85*) down to a concentration of about 10^5 particles/ml.

7. *Rabies*

The classical method for detecting neutralizing antibodies for rabies is seroneutralization using mice as subjects; however, the results obtained are variable and the method is time-consuming (10–21 days). Wiktor et al. improved the reproducibility and speed of the method by using tissue culture and inhibition immunofluorescence (*143*), while Atanasiu & Perrin developed an immunoenzymatic micromethod for the titration of antirabies antibodies using as antigens either the rabies virus or its glycoprotein (*2*). Comparable results are then obtainable in a few hours only (*130*).

Rabies vaccine and laboratory-adapted rabies virus both replicate in cell culture to give high titres. However, wild strains of the virus grow poorly in cell culture and it is difficult to produce the amount of virus required for large-scale screening. Smith et al. therefore developed a procedure in which cells infected with γ-irradiated rabies virus rather than the purified virus are used as target antigens (*123*). A single freeze-thawing cycle allows the reaction of the antibody with both internal and external (nucleocapsid and membrane) viral antigens.

8. *Arboviruses and viral fevers*

Attempts to isolate arboviruses from patients are rarely successful and diagnosis usually depends on serological testing. Though complement fixation, haemagglutination inhibition, and neutralization may sometimes be useful in this respect, they have important disadvantages (*16*). Immunoassays, specifically antibody-capture enzyme immunoassay, have proved extremely useful for this purpose and are widely used, e.g., for Japanese encephalitis (*13, 14, 19, 60, 148*), tick-borne encephalitis (*48, 49, 115*), St. Louis encephalitis (*98, 144*), and yellow fever (*28*).

Kurstak et al. detected arbovirus antigens in infected cells using the immunoperoxidase method (*82*). Use of this method also permitted McLean to demonstrate the intracytoplasmic replication of California encephalitis virus in both domestic and wild mosquitos (*91*).

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 viraemic 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. developed an ELISA test for β-propiolactone-inactivated sera that provides a definitive diagnosis within a few hours (*103*).

CONCLUSIONS

The introduction of the immunoperoxidase technique has been exceedingly important for diagnostic purposes (*71, 72*), and its use to detect molecules immobilized on a solid phase (most frequently polystyrene) has revolutionized research and diagnosis in virology. Early methods were adopted because they were simple, cheap, fast, and sensitive; however, with the advent of highly specific reagents, such as monoclonal antibodies, the specificity of the methods increased considerably. Moreover, techniques were developed for the detailed study of the immune response to viral infections. It had long been suspected that immunoglobulin class and subclass responses to viruses were important since these immunoglobulins differ in their biological properties. The introduction of class-capture enzyme immunoassays permitted a much better follow-up of IgM and IgG immune responses than had been possible with the classical methods and exposed many erroneous assumptions about the relation between primary infection and IgM response, particularly for viruses that lack antigenic uniformity (*92*), such as enteroviruses, adenoviruses, parainfluenzaviruses, and some togaviruses. Class-capture assays are also increasingly used for studying the response of the IgG subclass 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. In contrast, human IgG_2 antibodies are almost exclusively directed against polysaccharides, e.g., lipopolysaccharides of bacteria.

The trend towards 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 defence systems. In addition, an increasing number of commercial EIA kits will probably become available for the detection of infectious agents or their antibodies. Also it can be expected that the assays will become more rapid and simpler.

Recent advances in recombinant DNA technology have enabled nucleic acid probes to be used in the diagnosis of human viral diseases. However, wide

application of these probes will only occur if non-radioactive detectors are used to determine whether the probes have hybridized to the sample nucleic acid. This methodology is suitable both for quantitation and for localization purposes and is a valuable adjunct to enzyme immunoassay.

The examples described here illustrate the application of EIA or DNA probes to the study of viral diseases that were previously difficult to diagnose. However, in 1982 alone approximately 700 papers were published with "ELISA" in their title, and this attests both to the popularity of the technique and to the difficulties of reviewing this topic comprehensively.

ACKNOWLEDGEMENTS

Research projects carried out by Professor E. Kurstak were supported by the Medical Research Council of Canada, the National Institute of Cancer, and the Natural Sciences and Engineering Research Council of Canada.

RÉSUMÉ

ÉPREUVES IMMUNOENZYMATIQUES ET TECHNIQUES APPARENTÉES POUR LE DIAGNOSTIC VIROLOGIQUE AU LABORATOIRE

Les épreuves immunoenzymatiques (IE), qui représentent un des meilleurs outils du diagnostic virologique, ont été considérablement améliorées par la mise au point de nouveaux réactifs (anticorps monoclonaux, enzymes, conjugués, substrats). Les épreuves IE sont complémentaires des nouvelles sondes nucléotidiques qui permettent de mieux détecter des molécules comme les proto-oncogènes. Deux types d'épreuves IE peuvent être distingués du point de vue théorique. D'une part, un excès de la molécule détectrice marquée peut être utilisé, ce qui rend l'épreuve extrêmement sensible; en effet, d'après la loi d'action de masse, la réactivité dépend du produit des concentrations de l'antigène et de l'anticorps et non des concentrations individuelles. D'autre part, l'influence des constantes d'affinité est annulée, ce qui diminue la spécificité de la réaction.

Dans le deuxième type d'épreuve, des systèmes antigènes-anticorps différents sont présents qui font intervenir la constante d'affinité et augmentent la spécificité tout en diminuant la sensibilité (les épreuves compétitives sont souvent de ce type). Il est souvent assez difficile d'adapter les réactifs aux différentes exigences des différentes épreuves. Dans le cas des sondes nucléotidiques, les enzymes marquées à la streptavidine n'ont pas encore complètement remplacé les marqueurs radioactifs plus sensibles.

Les épreuves IE ont conduit à une meilleure compréhension de la réponse immunitaire de type humoral, par exemple en ce qui concerne les variations de la réponse IgM au cours du temps. Ces épreuves, basées sur la capture d'immunoglobulines d'une classe particulière, permettent d'identifier les classes des anticorps antiviraux. L'adsorption non spécifique des IgM est supérieure à celle des IgG mais peut être évitée par une dilution appropriée. Il existe des trousses commerciales pour les épreuves IE pour un grand nombre d'infections virales (hépatite, rotavirus, SIDA, rubéole, herpès). La préparation de ces réactifs au laboratoire est cependant assez simple et il est parfois possible d'obtenir une qualité supérieure. Certains échantillons deviennent faussement positifs par une digestion rapide de l'antigène, phénomène qui peut être évité par l'utilisation d'inhibiteurs des protéases. L'application des épreuves immunoenzymatiques au diagnostic de diverses infections virales d'importance médicale est discutée ainsi que différentes façons d'augmenter leur spécificité et de diminuer les réactions croisées. L'utilisation de ces épreuves augmentera certainement à l'avenir, surtout pour des virus qui ne peuvent être cultivés (hépatite, parvovirus) ou qui doivent être inactivés. On peut aussi s'attendre à la mise au point d'épreuves immunoenzymatiques homogènes permettant d'éliminer la phase solide de coût élevé, et qui rendront ce type d'épreuve encore plus rapide.

REFERENCES

1. AL-KAISSI, E. N. & MOSTRATOS, A. *Journal of virological methods*, **4**: 353 (1982).
2. ATANASIU, P. & PERRIN, P. *Annales de microbiologie*, **130A**: 257 (1979).
3. BEARDS, G. M. ET AL. *Journal of clinical microbiology*, **19**: 248 (1984).
4. BEST, J. M. ET AL. In: Kurstak, E., ed. *Applied virology*. New York, Academic Press, 1984, pp. 221–230.
5. BEST, J. M. ET AL. *Journal of virological methods*, **8**: 99 (1984).

6. BLUM, H. E. ET AL. In: Vijas, G. N. et al., ed. *Viral hepatitis and liver disease*. Orlando, FA, Grune and Shatton, 1984, p. 634.
7. BORNKAMM, G. W. ET AL. *Current topics in microbiology and immunology*, **104**: 287 (1983).
8. BOTELER, W. L. ET AL. *Journal of clinical microbiology*, **17**: 814 (1983).
9. BOUÉ, A. ET AL. *Lancet*, **1**: 1251 (1971).
10. BRANDSMA, J. E. & MILLER, G. *Proceedings of the National Academy of Sciences of the USA*, **77**: 6851 (1980).
11. BRANDT, C. D. *Journal of clinical microbiology*, **18**: 71 (1983).
12. BRIGATI, D. J. *Virology*, **126**: 32 (1983).
13. BURKE, D. S. & NISALAK, A. *Journal of clinical microbiology*, **15**: 353 (1982).
14. BURKE, D. S. ET AL. *Journal of clinical microbiology*, **16**: 1034 (1982).
15. BURRELL, C. J. ET AL. *Hepatology (Suppl.)* **2**: 85 (1982).
16. CALISHER, C. H. & POLAND, J. D. In: Monath, T. P., ed. *St. Louis enchephalitis*. Washington, DC, American Public Health Association, 1980, p. 371.
17. CAPPEL, R. ET AL. *Archives of virology*, **58**: 253 (1978).
18. CENTERS FOR DISEASE CONTROL. *Journal of infectious diseases*, **137**: 817 (1978).
19. CHANG, H-C. ET AL. *Journal of virological methods*, **9**: 143 (1984).
20. CHOU, S. & MERIGAN, T. C. *New England journal of medicine*, **308**: 921 (1983).
21. CLEWLEY, J. P. *Journal of medical virology*, **15**: 173 (1985).
22. COLEMAN, R. M. ET AL. *Journal of clinical microbiology*, **8**: 287 (1983).
23. COULSON, B. S. & HOLMES, I. H. *Journal of medical virology*, **8**: 165 (1984).
24. CRADOCK-WATSON, J. E. ET AL. *Journal of hygiene*, **76**: 109 (1979).
25. CRANAGE, M. P. & GARDNER, P. S. *Journal of medical virology*, **5**: 161 (1980).
26. CUKOR, G. ET AL. *Journal of clinical microbiology*, **19**: 888 (1984).
27. DE JONG, J. C. ET AL. *Journal of medical virology*, **11**: 215 (1983).
28. DUEBEL, V. ET AL. *American journal of tropical medicine and hygiene*, **32**: 565 (1983).
29. DUERMEYER, W. & VAN DER VEEN, J. *Lancet*, **2**: 684 (1978).
30. ENDERS, G. (1984). *Medical laboratory sciences, suppl. 1*, 58 (1986).
31. FEINMAN, S. V. *Journal of virological methods*, **8**: 199 (1985).
32. FLEWETT, T. H. & BABIUK, L. A. In: Kurstak, E. & Marusyk, R. G., ed. *Control of virus diseases*. New York, Dekker, 1984, pp. 57–65.
33. FLORES, J. ET AL. *Lancet*, **1**: 555 (1983).
34. FLOWER, A. J. E. *British medical journal*, **2**: 220 (1977).
35. FORGHANI, B. ET AL. *Journal of virological methods*, **5**: 317 (1982).
36. FORGHANI, B. ET AL. *Journal of clinical microbiology*, **18**: 632 (1983).
37. GADKARI, D. A. ET AL. *Journal of virological methods*, **10**: 215 (1985).
38. GILLJAM, G. ET AL. *Journal of virological methods*, **10**: 203 (1985).
39. GISPEN, R. ET AL. *Clinical and experimental immunology*, **22**: 431 (1975).
40. GOLDWATER, P. N. ET AL. *Journal of virological methods*, **4**: 9 (1982).
41. GREENBERG, H. B. ET AL. *Journal of virology*, **37**: 994 (1981).
42. GROM, J. & BERNARD, S. *Journal of virological methods*, **10**: 135 (1985).
43. HAMMER, S. M. ET AL. *Journal of infectious diseases*, **141**: 436 (1980).
44. HAMMOND, G. W. ET AL. *Journal of clinical microbiology*, **16**: 53 (1982).
45. HAMPAR, B. ET AL. *Journal of clinical microbiology*, **21**: 496 (1985).
46. HARCOURT, G. C. ET AL. *Journal of infectious diseases*, **142**: 145 (1980).
47. HARRISON, T. J. ET AL. *Journal of virological methods*, **6**: 295 (1983).
48. HEINZ, F. X. ET AL. *Journal of clinical microbiology*, **14**: 141 (1981).
49. HOFMAN, H. ET AL. *Journal of general virology*, **42**: 505 (1979).
50. HOGG, R. J. & DAVIDSON, G. P. *Australian paediatric journal*, **18**: 184 (1982).
51. HORNSLETH, A. ET AL. *Journal of of medical virology*, **10**: 273 (1982).
52. HORNSLETH, A. ET AL. *Journal of medical virology*, **13**: 149 (1984).
53. HOVI, T. ET AL. *Journal of virological methods*, **5**: 45 (1982).
54. HYMAN, R. W. ET AL. *Journal of molecular biology*, **77**: 189 (1973).
55. IDA, S. ET AL. *Journal of general virology*, **64**: 533 (1983).
56. ISAAC, M. & PAYNE, R. A. *Journal of medical virology*, **10**: 55 (1982).
57. JULKUNEN, I. *Journal of medical virology*, **14**: 177 (1984).
58. KALICA, A. R. ET AL. *Virology*, **112**: 385 (1981).
59. KANGRO, H. O. *British journal of experimental pathology*, **61**: 512 (1980).
60. KONISHI, E. & YAMAOKA, M. *Kobe journal of medical sciences*, **28**: 7 (1982).
61. KHAN, M. W. ET AL. *Journal of clinical microbiology*, **16**: 115 (1982).
62. KRAAIJEVELD, C. A. ET AL. *Journal of clinical microbiology*, **12**: 493 (1980).
63. KRAMER, S. M. & CREMER, N. E. *Journal of virological methods*, **8**: 255 (1984).
64. KRAUSE, P. J. ET AL. *Journal of pediatrics*, **102**: 259 (1983).
65. KRISHNA, R. V. ET AL. *Journal of clinical microbiology*, **12**: 46 (1980).
66. KRONVALL, G. & WILLIAMS, R. *Journal of immunology*, **103**: 828 (1969).
67. KRYGER, P. *Journal of virological methods*, **10**: 283 (1985).
68. KUNZ, C. & HOFMANN, H. *Zentralblatt für Bakteriologie, Reihe A*, **218**: 273 (1971).

69. KURSTAK, E. *Enzyme immunodiagnosis*. New York, Academic Press, 1986.
70. KURSTAK, E. *Bulletin of the World Health Organization*, **63**: 793-811 (1985).
71. KURSTAK, E. In: Maramorosch, K. & Koprowski, H., ed. *Methods in virology. vol. 5*. New York, Academic Press, 1979, pp. 423-444.
72. KURSTAK, E. ET AL. *Comptes rendues hebdomadaires des séances de l'Académie des Sciences, série D*, **268**: 2309 (1969).
73. KURSTAK, E. ET AL. *Journal of medical virology*, **2**: 189 (1978).
74. KURSTAK, E. & KURSTAK, C. In: Kurstak, E. and Morrisset, R., ed. *Viral immunodiagnosis*, New York, Academic Press, 1974, pp. 3-30.
75. KURSTAK, E. ET AL. *Developments in biological standardization*, **52**: 187 (1982).
76. KURSTAK, E. ET AL. In: Kurstak, E. & Kurstak, C., ed. *Comparative diagnosis of viral diseases, vol. IV*, New York, Academic Press, 1981, pp. 105-148.
77. KURSTAK, C. ET AL. *Annales de virologie*, **133E**: 187 (1982).
78. KURSTAK, E. ET AL. In: Kurstak, E. & Kurstak, C., ed. *Comparative diagnosis of viral diseases, vol. II*. New York, Academic Press, 1977, pp. 404-448.
79. KURSTAK, E. ET AL. In: Kurstak, E., ed. *Applied virology*, New York, Academic Press, 1984, p. 518.
80. KURSTAK, E. In: Kurstak, E. & Marusyk, R. G., ed. *Control of virus diseases*, New York, Dekker, 1984, pp. 477-500.
81. KURSTAK, E. ET AL. *Annals of the New York Academy of Sciences*, **254**: 369 (1975).
82. KURSTAK, E. ET AL. In: Kurstak E. et al., ed. *Invertebrate systems* in vitro. Amsterdam, Elsevier Biomedical Press, 1980, pp. 365-373.
83. LANGER, P. R. ET AL. *Proceedings of the National Academy of Sciences of the USA*, **78**: 6633 (1982).
84. LINDE, A. ET AL. *Infection and immunity*, **42**: 237 (1983).
85. MACNAUGHTON, M. R. ET AL. *Journal of medical virology*, **11**: 319 (1983).
86. MADORE, H. P. ET AL. *Journal of clinical microbiology*, **18**: 1345 (1983).
87. MANIATIS. T. ET AL. *Molecular cloning: a laboratory manual*. New York, Cold Spring Harbor Laboratory, 1982.
88. MARUSYK, R. G. In: Kurstak, E., ed. *Applied virology*. New York, Academic Press, 1984, pp. 211-219.
89. MASON, W. S. ET AL. *Proceedings of the National Academy of Sciences of the USA*, **79**: 3997 (1982).
90. MCLEAN, B. ET AL. *Journal of clinical microbiology*, **12**: 314 (1980).
91. MCLEAN, D. M. In: Kurstak, E., ed. *Arctic and tropical arboviruses*. New York, Academic Press, 1979, pp. 7-19.
92. MEURMAN, O. *Current topics in microbiology and immunology*, **104**: 101 (1983).
93. MEURMAN, O. ET AL. *Journal of medical virology*, **14**: 67 (1984).
94. MEURMAN, O. ET AL. *Journal of medical virology*, **17**: 61 (1984).
95. MEURMAN, O. H. & ZIOLA, B. R. *Journal of clinical pathology*, **31**: 483 (1978).
96. MILAN, A. & STIGBRAND, T. *Clinical chemistry*, **27**: 2018 (1981).
97. MILLER, E. ET AL. *Lancet*, **2**: 781 (1982).
98. MONATH, T. P. ET AL. *Journal of clinical microbiology*, **20**: 784 (1984).
99. MORELL, A. ET AL. *Infection and immunology*, **39**: 565 (1983).
100. MORGAN, E. M. & RAPP, F. *Bacteriological reviews*, **41**: 636 (1977).
101. MORGAN-CAPNER, P. ET AL. *Journal of hygiene*, **90**: 407 (1983).
102. MURPHY, B. R. ET AL. *Journal of clinical microbiology*, **13**: 554 (1981).
103. NIKLASSON, B. S. ET AL. *Journal of clinical microbiology*, **20**: 239 (1984).
104. O'BEIRNE, A. ET AL. In: *Rapid methods and automation in microbiology*. Washington, DC, American Society for Microbiology, 1982, p. 139.
105. PAPAEVANGELOU, G. ET AL. *Journal of medical virology*, **13**: 393 (1984).
106. PARKINSON, A. J. ET AL. *Journal of clinical microbiology*, **15**: 538 (1982).
107. PATTISON, J. R. ET AL. *Journal of medical microbiology*, **9**: 355 (1976).
108. PAULS, F. P. & DOWDLE, W. R. *Journal of immunology*, **98**: 941 (1967).
109. RAPICETTA, M. ET AL. *Journal of virological methods*, **6**: 303 (1983).
110. RAWLS, W. E. ET AL. *Journal of immunology*, **104**: 599 (1979).
111. RECCHIA, S. ET AL. *Pathologica*, **73**: 773 (1981).
112. REINER, M. & WECKER, E. *Medical microbiology and immunology*, **169**: 237 (1981).
113. RICE, G. P. A. ET AL. *Journal of infectious diseases*, **147**: 1055 (1983).
114. RIGBY, P. W. J. *Journal of molecular biology*, **113**: 237 (1977).
115. ROGGENDORF, M. ET AL. *Journal of medical virology*, **7**: 41 (1981).
116. ROGGENDORF, M. ET AL. *Journal of virological methods*, **4**: 27 (1982).
117. SARKKINEN, H. K. ET AL. *Journal of clinical microbiology*, **13**: 258 (1981).
118. SCHLUEDERBERG, A. *Nature*, **205**: 1232 (1965).
119. SCOTTO. J. ET AL. *Gut*, **24**: 618 (1983).
120. SEQUIERA, L. W. ET AL. *Lancet*, **2**: 609 (1979).
121. SHATTOCK, A. G. & MORGAN, B. M. *Journal of medical virology*, **13**: 73 (1984).
122. SMEDILE, A. ET AL. *Journal of medical virology*, **9**: 131 (1982).
123. SMITH, J. S. ET AL. *Journal of clinical microbiology*, **19**: 267 (1984).
124. SPECTOR, S. A. *Lancet*, **1**: 378 (1983).
125. STALHANDSKE, P. & PETTERSSON, U. *Journal of clinical microbiology*, **15**: 744 (1982).
126. STALLMAN, N. D. ET AL. *Medical journal of Australia*, **2**: 629 (1974).
127. STEECE, R. C. ET AL. *Journal of clinical microbiology*, **19**: 923 (1984).
128. STREET, J. E. ET AL. *Journal of virology*, **43**: 369 (1982).
129. SUNDQVIST, V-A. ET AL. *Journal of clinical microbiology*, **20**: 94 (1984).

130. SUREAU, P. ET AL. *Comparative immunology, microbiology, and infectious diseases,*, **5**: 143 (1982).
131. THOMAS, H. C. ET AL. *Journal of virological methods*, **10**: 291 (1985).
132. TIJSSEN, P. In: Burdon, H. & van Knippenberg, P. H., ed. *Laboratory techniques in biochemistry and molecular biology*, Amsterdam, Elsevier, 1985.
133. TURNER, R, ET AL. *Journal of clinical microbiology*, **15**: 824 (1982).
134. UKKONEN, P. ET AL. *Journal of clinical microbiology*, **11**: 319 (1980).
135. VAN LOON, A. M. ET AL. *Journal of clinical microbiology*, **13**: 416 (1981).
136. VAN LOON, A. M. ET AL. *Journal of clinical microbiology*, **21**: 558 (1985).
137. VAN NIEUWKOOP, J. ET AL. *International archives of allergy and applied immunology*, **67**: 61 (1982).
138. VEJTORP, M. *Journal of virological methods*, **1**: 1 (1980).
139. VESTERGAARD, B. F. *Developments in biological standardization*, **52**: 95 (1982).
140. VUORIMAA, T. O. ET AL. *Journal of medical virology*, **2**: 271 (1978).
141. WELLIVER, R. C. ET AL. *Journal of pediatrics*, **96**: 808 (1980).
142. WHELCHEL, J. D. ET AL. *Transplantation*, **28**: 443 (1979).
143. WIKTOR, T. J. ET AL. *Journal of immunology*, **97**: 822 (1973).
144. WOLFF, K. L. ET AL. *Journal of clinical microbiology*, **14**: 135 (1981).
145. WOLTERS, G. ET AL. *Journal of clinical pathology*, **29**: 873 (1976).
146. WOLTERS, G. ET AL. *Journal of virological methods*, **10**: 299 (1985).
147. YOLKEN, R. H. In: Tyrrell, D. A. & Kapikian, A. Z., ed. *Virus infections of the gastrointestinal tract*. New York, Marcel Dekker, 1982, p. 51.
148. ZHANG, Y-H. ET AL. *Journal of virological methods*, **9**: 45 (1984).
149. MCKEATING, J. A. ET AL. *Journal of medical virology*, **18**: 341 (1986).
150. WREGHITT, T. G., ET AL. *Journal of medical virology*, **18**: 119 (1986).
151. FLANDERS, R. T. ET AL. *Journal of medical virology*, **19**: 1 (1986).