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The Diagnosis of Human Immunodeficiency Virus Infection: Progress in Less than Five Years

FRANK J. MICHALSKI, Ph.D.

Diagnostic Virology Laboratory, MetPath, Inc., Corning Laboratory Sciences, Teterboro, New Jersey

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Diagnostic tests for human immunodeficiency virus first became commercially available in 1985, only two years after the virus was discovered. In the short period of time since then, we have witnessed improvements in antibody detection methods, refinements in culture techniques, and the introduction of antigen and nucleic acid detection methods, including the polymerase chain reaction. These diagnostic tools as well as their advantages and disadvantages are reviewed in this report.

INTRODUCTION

Routine screening and diagnosis of human immunodeficiency virus type 1 (HIV-1) started in 1985 with the introduction of the enzyme-linked immunosorbent assay (ELISA) for antibody detection, only two years after the initial isolation of the etiologic agent for the acquired immune deficiency syndrome (AIDS). Since then, dramatic improvements in methods of diagnosis have rapidly been introduced, such as refined isolation and antibody detection methods, antigen detection, and nucleic acid detection (Table 1). This paper reviews the developments that have occurred from the viewpoint of a director of a large diagnostic virology laboratory.

ISOLATION AND CULTIVATION OF HIV-1

Original Discovery

In the first reports of isolation of HIV-1 [1,2], T cells from the peripheral blood of an AIDS patient and from the lymph node biopsy of a homosexual with generalized lymphadenopathy were cultivated *in vitro* in medium containing human T-cell growth factor (TCGF), which is now referred to as interleukin 2. Virus was visualized in these cultured cells by electron microscopy, and virus-specific reverse transcriptase (RT) was detected in the culture medium. The virus was then transmitted *in vitro* to T cells derived from cord blood of newborns or healthy adult donors, using co-cultivation techniques. Barre-Sinoussi et al. [1] included phytohemagglutinin (PHA) in the culture medium for three days to stimulate the T cells to divide, antiserum to human alpha interferon to neutralize endogenous interferon, and polybrene (2 mcg/ml) to aid virus adsorption to uninfected T cells.

Abbreviations: AIDS: acquired immune deficiency syndrome ARC: AIDS-related complex CDC: Centers for Disease Control CPE: cytopathic effect DBS: dried blood spots ELISA: enzyme-linked immunosorbent assay HIV-1: human immunodeficiency virus type 1 PBL: peripheral blood lymphocytes PCR: polymerase chain reaction PHA: phytohemogglutinin RT: reverse transcriptase TCGF: T-cell growth factor WB: Western blot test

Address reprint requests to: Frank J. Michalski, Ph.D., Diagnostic Virology Laboratory, MetPath, Inc., Corning Laboratory Sciences, Teterboro, NJ 07608

TABLE 1
Diagnostic Techniques for HIV-1

Method	Initial Use	Description	Current Status (1988–1989)	Reference Cited
Isolation and culture of HIV-1	1983	Growth and detection of virus in living cells	Clinical diagnosis	[1,2]
Antibody detection				
ELISA using cell culture antigen	1985	Detection of antibody	Routine blood screening and clinical diagnosis	[26]
Western blot	1985	Confirmation of antibody detected	Routine confirmation of ELISA	[27,28]
Immunofluorescence, hemagglutination, radioimmunoprecipitation, and so on	1985	Detection of antibody	Limited use	[29,30,31,32,33,34,35]
ELISA using recombinant antigen or synthetic peptide	1986	Detection of antibody	Research	[43,44]
Antigen detection	1986	Detection of viral antigen	Research	[50,51,52,53]
Nucleic acid detection	1987	Detection of viral nucleic acid	Research	[57,58,59]

Problems and Difficulties

Shortage of Trained Personnel The method of co-cultivation with fresh human peripheral blood lymphocytes (PBL) and detection of virus by assay for RT were unfamiliar techniques to most diagnostic virologists [3,4]. Only researchers who had worked on avian or murine retroviruses were proficient in these techniques [5,6]. Thus, in 1985, the Centers for Disease Control (CDC) offered courses to train technologists in HIV-1 culture [7] and the California State Health Department produced a guide for culture techniques [8]. This guide helped many laboratories to become established; however, since HIV-1 infections can lead to AIDS with its high mortality rate, few technologists were willing to work with this virus. Subsequent reports of a few cases of laboratory-acquired infection showed that the danger was real, although slight [9,10].

Turnaround Time In addition to the specialized techniques and the risk of working with HIV-1, the isolation of HIV is a lengthy process. Although the majority of cultures for HIV-1 become positive within about ten days, cultures must be maintained and tested for at least four weeks before being considered negative [7,11].

Lack of Established Cell Lines for Culture One of the early breakthroughs in HIV research was the discovery of the H9 cell line, which was capable of replicating HIV-1 to high titer [3]. Unfortunately, early work at CDC and elsewhere showed that this cell line and others were not as sensitive as primary PBL for initial isolation of

HIV-1 from clinical specimens [7,12]. The logistical problems of isolating HIV from clinical specimens involved, first, obtaining fresh PBL from healthy donors (negative for antibody to HIV-1 and hepatitis B); second, establishing the PBL in culture by stimulation with PHA for three days; and, finally, maintaining the stimulated cells in culture in preparation for co-cultivation attempts. Stimulated PBL were also needed for the weekly refeeding of the ongoing cultures.

Expense Isolation of HIV-1 is an expensive procedure. Therefore, the use of this test as a diagnostic tool can only be justified for highly specific reasons. Laboratories routinely charge $400 to $500 for the service. The reasons for this high cost include the need for expensive equipment and reagents, as well as skilled technical help, and the labor-intensive nature of the technique.

Culture requires a separate isolation room with a biological safety cabinet (Class II), as well as a CO_2 incubator for culture of lymphocytes, a $-70°C$ freezer, a scintillation counter for RT, and a liquid nitrogen tank for storage of cells.

Procurement of human PBL for co-cultivation can be costly and difficult. The medium needed for culture of the lymphocytes must be supplemented with fetal bovine serum, interleukin 2, and anti-human interferon. Reagents for the RT assay include template primer and ^3H-labeled deoxythymidine triphosphate. Proper disposal of radioactive isotopes is also expensive. Finally, a highly skilled technologist is essential, and cultures must be maintained for at least four weeks with frequent refeeding and testing for virus.

Recovery Rate In early attempts, HIV-1 could be isolated from only 30–89 percent of antibody-positive patients [13,14]. Furthermore, virus recovery varied with the stage of disease, with virus harder to isolate in the early stages when culture would be of most use. Recent reports indicate that it is possible to cultivate HIV-1 from close to 100 percent of those patients in whom the presence of detectable antibody to HIV-1 proves infection with this virus [13,14,15].

Recent Developments

Use of Frozen PBL The finding that PBL from normal donors, as well as those from patients' specimens, could be frozen and thawed before use has helped to ease the logistical problems of availability and transport of PBL. Frozen cells from normal donors, however, are somewhat less sensitive for isolation and therefore should be used only when fresh cells are not available.

Balachandran et al. [16] reported that the susceptibility to HIV-1 infection was similar for normal human donor PBL that had been stimulated with PHA for 72 hours and either used fresh or after having been frozen in cryopreservative fluid and thawed. They also found that PBL that were first cryopreserved and thawed, then subsequently stimulated with PHA for 72 hours, also retained susceptibility to HIV-1 infection.

In clinical trials, HIV-1 was isolated from 15 of 15 AIDS patients with cryopreserved PBL. In contrast, HIV-1 was isolated from 11 of 13 asymptomatic HIV-1 seropositive men using fresh PBL, but only 3 of 13 using cryopreserved PBL [16]. Further studies are needed to determine whether cultures for HIV-1 seropositive asymptomatic patients must only be performed with fresh PBL.

Gallo et al. [11,17] compared isolation rates of HIV-1 from Ficoll-Hypaque separated PBL from patients' specimens, before and after three freeze-and-thaw cycles in RPMI 1640 medium containing 10 percent fetal bovine serum and 10 percent

dimethylsulfoxide. There was no general effect on isolation rate, suggesting that, in the case of patients' samples, frozen PBL were as suitable as fresh PBL for HIV-1 isolation attempts. Thus the problem of getting heparinized blood from the patient to the laboratory within 24 hours of collection was solved by using frozen cells. After separated PBL are frozen, they can easily be shipped to the laboratory on dry ice and thawed for co-cultivation attempts whenever it is most convenient for the laboratory.

Micro-Techniques for Culture Castro et al. [14] reported that cultures in smaller vessels with a high cell density per unit surface area yielded better virus recovery than cultures in larger vessels and required fewer cells. This latter feature was particularly important in pediatric patients, from whom only small amounts of blood were available. The majority of positive cultures (62.5 percent) were detected within 4–11 days of incubation, and the sensitivity of the method was such that one infected lymphocyte in one million would be detected. The use of smaller culture vessels and fewer cells also helped lower the cost of cells and medium.

Improved Detection of Virus in Culture Supernate After cultures have been initiated, they must be tested once or twice a week for the presence of virus. The standard method of HIV detection in culture supernate has been the assay for the viral enzyme RT, instead of detection of viral cytopathic effect (CPE). Multinucleated giant cells form in the cultured infected lymphocytes after four to six days [12], but this process has not been a sensitive or consistent method of detecting the virus.

Two drawbacks to the RT assay have been specificity (i.e., ability to distinguish HIV-1 RT from cellular polymerases and from RT of other retroviruses, HTLV-1 and II, and HIV-2 [12]) and the difficulty and cost of working with and disposing of radioisotopes (^{3}H). It has also been tedious to perform; however, some improvements have been made. Spira et al. [18] reported that a micro method for assay of RT was equal to the macro method and saved both time and reagents. Lee et al. [19] developed a RT assay that gave a 20- to 40-fold increase in enzyme activity over the current method for detection and was sensitive enough to detect as few as 250 HIV-1 particles in culture medium.

Yet, in spite of these improvements, the RT assay remains difficult, and a better assay for virus detection was sought. Feorino et al. [20] reported that an antigen capture ELISA was 100-fold more sensitive than the RT assay in detecting HIV-1 in cell culture. The test was specific only for HIV-1 and gave positive results more quickly, with an average time of 5.9 days versus 9.6 days for the RT assay. Lee et al. [21] reported in a subsequent study that the antigen capture ELISA was sensitive enough to detect 75 virus particles as compared to 130 virus particles for the RT assay.

Viscidi et al. [22] found that 50 percent of virus-containing cultures were identified within nine days by antigen capture ELISA but within 14 days by the RT assay. Also, in cultures sampled on several days, RT activity was detected intermittently, whereas antigen levels did not decline after initial appearance. Jackson et al. [13] used the antigen capture ELISA in place of the RT assay for HIV-1 cultures and showed improved sensitivity. Burger et al. [23] also found that antigen capture ELISA was superior to RT for monitoring HIV-1 cultures in clinical trials.

As a result of these studies and others [24,25], when only HIV-1 is being sought in cell cultures, the antigen capture ELISA has now become the method of choice over RT since it produces more sensitive and rapid results. If retroviruses other than HIV-1 are suspected, then the RT assay must also be performed.

DETECTION OF HIV ANTIBODY

Advantages

Several types of serological tests for HIV-1 antibody are available (Table 1), including ELISA [26], Western blot [27,28], immunofluorescence [29,30,31,32], radioimmunoprecipitation [33,34], and hemagglutination [35]. All these tests can be performed rapidly, with the most commonly used ELISA requiring two to five hours to complete. In addition, all of the eight approved ELISAs have a sensitivity and specificity of over 99 percent. The ELISA is also easily automated, which saves personnel time and insures accurate pipetting and objective reading of results. Furthermore, because it can be automated, the ELISA can be done at low cost.

Disadvantages

Antibodies to HIV-1 develop about 8–12 weeks after infection, creating a window when early infection is not detectable by antibody [37,38]. Also, late in infection after disease has developed, antibody to p24 decreases; however, this latter finding may be useful in prognosis [39]. The sensitivity and specificity of ELISA is dependent upon the prevalence of infection in the population tested, and for diagnostic purposes a supplemental test such as Western blot, immunofluorescence, or the recombinant ELISA (Table 1) is needed to confirm positive results. In newborns, the diagnosis of HIV-1 infection by antibody tests can be complicated, because maternal antibodies may cross the placenta and give a positive result even when the baby is not infected. A disadvantage of several antibody tests, such as Western blot and immunofluorescence, is that interpretation is dependent upon subjective criteria.

Recent Developments

Standardization of the Western Blot Better standardization of the Western blot (WB) procedure is anticipated through better dissemination of information on the procedure and more consensus on interpretation of banding patterns [42]. In addition, several commercial semi-automated WB procedures are now available.

ELISAs Using Recombinant Protein or Synthetic Peptides HIV-1 grown in human lymphocytes is used as antigen for the standard ELISA. Thus, reaction of antibody in the patients' serum to human cellular antigens contaminating the viral antigen preparation is possible, giving rise to false-positive results. This problem has been overcome by the use of recombinant viral proteins made in bacteria or yeast [43] or synthetic peptides [44] as antigen in the ELISA.

Screening of Dried Blood Spots (DBS) Human whole blood dried on to filter paper represents a potential important sample collection method for HIV antibody testing. Reports so far show that testing of DBS from both adults and neonates is a suitable technique for HIV-1 antibody screening using commonly available commercial ELISAs [45].

Quick Membrane ELISA Several commercial companies (Abbott Laboratories, Genetic Systems) are testing in small plastic format membrane ELISAs that give results in minutes. They are easy to perform and are read by visualization of a color pattern on the membrane. This technique allows rapid testing in emergency rooms and in countries where spectrophotometers are not readily available.

ANTIGEN TESTING

Advantages

More Rapid than Culture The current status of antigen testing has been recently summarized [46]. In 1986, commercial tests became available for direct detection of HIV-1 antigen in serum, plasma, and cerebrospinal fluid (CSF), using the ELISA technique. A blocking antibody (so-called neutralization test) should be used to confirm positive results. If overnight incubation is used, the ELISA takes 24–30 hours to complete as compared to four weeks for HIV-1 isolation in culture. Recently introduced antigen detection kits do not require overnight incubation; thus the time can be shortened to three to five hours. (Direct detection of HIV-1 antigen in patients' samples should not be confused with detection of HIV-1 antigen in the supernate of HIV-1-infected cultures, as was discussed earlier in this review.)

Indicator of Active Infection In contrast to antibody tests, a positive direct antigen detection indicates active HIV-1 infection. Conversely, in healthy individuals in low-risk groups with indeterminate WB results, a negative antigen test may provide some reassurance.

Early Detection of Infection Antigen detection allows the diagnosis of HIV-1 infection prior to antibody seroconversion, which may take longer than one year from the time of exposure [47]. Transient HIV-1 antigenemia has been linked to acute HIV infection before the appearance of HIV-1 antibody [48].

It is not known what percentage of individuals will express HIV antigen in serum during the interval between exposure and seroconversion.

Prognosis of Disease State Detection of HIV-1 antigen in serum has prognostic significance. In one report, asymptomatic individuals who were serum antigen-positive were 18 times more likely to progress to AIDS over a 21-month period [49]. A positive HIV-1 antigen test obtained from infants' serum is useful in confirming HIV infection [41]. An HIV-1 antigen test can also be helpful to differentiate between primary and HIV-associated immunodeficiency syndromes in childhood, since the ability to produce antibodies may be impaired in these children.

Monitoring Drug Treatment Monitoring HIV-1 antigen levels may be useful in measuring the antiviral effect of drug therapy. Mean HIV-1 antigen levels have been shown to decrease significantly over time in patients treated with zidovudine compared with placebo-treated controls [50].

Disadvantages

The sensitivity of the HIV-1 antigen assay is poor if one assumes that most if not all HIV-1 antibody-positive individuals are infected. The sensitivity of the test improves with clinical progression of disease, showing 4 percent positivity in asymptomatic patients, 56 percent positivity in AIDS-related complex (ARC) patients, and 70 percent positivity in AIDS patients [51,52]. The detectable limit of the assay has been reported to be between 50 and 100 pg/ml [53,54].

The reagents for antigen tests are more expensive than antibody testing and also more expensive than RT for HIV-1 detection in culture. Furthermore, the FDA has not yet (April 1989) approved this test for diagnostic use.

Recent Developments

New and revised antigen tests that claim a greater sensitivity have recently been introduced. Coulter Immunology claims a limit of detection of 1.0–1.56 pg per well for

HIV-1 and HIV-2 p24 core antigen. Further study is needed to evaluate the usefulness of these improved tests.

NUCLEIC ACID DETECTION

Advantages

Retroviruses are so named because of the RNA-dependent DNA polymerase (reverse transcriptase) that, during virus replication, synthesizes RNA into DNA. The DNA may then either be transcribed into RNA for the genetic material of new viruses or for translation into HIV-1 proteins. The HIV genome does not contain sequences homologous to normal human DNA [56] or to HTLV-I or HTLV-II DNA to any extent [57]. Thus, infected cells can be probed by labeled HIV-1 DNA or RNA sequences to detect HIV-1 nucleic acid by several hybridization techniques. Although direct detection of viral-specific nucleic acid in patient specimens is not especially sensitive, various methods of *in vitro* amplification of viral nucleic acid, such as polymerase chain reaction (PCR) [58,59,60], can make this test extremely sensitive. By selecting the proper gene probe, the test can be extremely specific. Using this methodology, viral DNA integrated into the cellular genome can also be detected to prove latent infection with HIV.

Disadvantages

Techniques for nucleic acid detection are technically difficult and remain research tools at the present time. In addition, much research must still be done to determine the significance of nucleic acid detection results.

CONCLUDING REMARKS

The number and types of tools that a laboratory can use for diagnosis of HIV-1 infection have increased over the past five years, making the selection of the proper tools in each case more difficult. At present, screening for HIV-1 infection is best performed by the ELISA for HIV-1 antibody, with confirmation of repeatedly reactive specimens by Western blot. When antibody results are indeterminate, resolution can be attempted by use of the recombinant antibody ELISA. The ELISA for antigen detection is indicated in cases of recent exposure to HIV-1, in neonates, to determine prognosis of high-risk antibody-positive patients, and to monitor drug therapy. If antigens tests are negative, virus isolation should be performed, since it is more sensitive. Nucleic acid hybridization is still used only as a research tool.

Since the true clinical significance of each diagnostic tool lags behind its development, the use of multiple tools will undoubtedly continue until the proper role for each is determined. In the next five years, the routine diagnosis of HIV-1 may be accomplished by as yet undiscovered methods.

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