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Laboratory Methods for Early Detection of Human Immunodeficiency Virus Type 1 in Newborns and Infants

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INTRODUCTION

Epidemiology of Perinatal HIV-1 Infections

The first cases of AIDS were reported to the Centers for Disease Control (CDC) in 1981 (14). Within a year, the first cases of AIDS in women and children were reported to the CDC (15, 16). As of May 1991, 179,136 cases of AIDS in all age groups had been reported in the United States (21). In 1991 alone, more than 30,000 cases of AIDS were reported to the CDC (23). From 1981 to 1990, AIDS has become one of the leading causes of death among women less than 45 years of age and in children 1 to 5 years of age (21, 22). Women of reproductive age represented 3.2% of all AIDS cases in 1981; by 1990, this value rose to 11.5% (22). Infants 0 to 4 years of age and children constituted 0% of AIDS cases in 1981 compared to 1.4% in 1990 (22).

A national survey of 38 states and the District of Columbia was begun in 1988 to assess the seroprevalence of human immunodeficiency virus type 1 (HIV-1) infection among childbearing women. The survey was done by blinded testing of blood specimens from newborns; the specimens were collected on filter paper for metabolic screening (45). In this study, the national seroprevalence rate among women who had given birth in 1989 was 0.15%. The highest rates (number positive per total number of specimens) were reported in New York (5.8/1,000), the District of Columbia (5.5/1,000), New Jersey (4.9/1,000), and Florida (4.5/1,000) (45). In the last decade in the United States, the trends in HIV-1 infection and AIDS in women have been towards a higher percentage of all cases of AIDS being women, a higher proportion of women acquiring the infection through heterosexual contact than through intravenous drug use, and the spread of infection in women from largely urban centers to smaller cities and rural areas (31).

HIV-1 Infections and Testing for HIV-1 during Pregnancy

Perinatal transmission of HIV-1 accounts for over 80% of all cases of AIDS in infants less than 1 year of age (36). The growing number of infected women who are in their reproductive years and the increasing percentage of HIV-1 infection occurring in women have led to the rise of AIDS in infants and children. Women of reproductive potential constitute 80% of all women with AIDS (20, 22). The rate of perinatal transmission has ranged from 15 to 40% (7, 66, 85, 86), although the most recent epidemiological data indicate that the transmission rate may be at the lower end of this range (35).

HIV testing of pregnant women is not universal. At best and in only a minority of prenatal clinics, pregnant women are screened regarding risks for acquiring HIV-1. Women identified as high risk are then offered HIV-1 antibody testing after counseling. Exhaustive and effective tactics for properly identifying pregnant women at risk for HIV-1 are lacking. One study, which screened mothers for HIV-1 antibody through cord blood testing, showed that none of the patients who tested positive had been identified during the prenatal period when a risk assessment screening procedure was used (94). In our own experience, it is not uncommon that the seropositive status of a mother is known because her <15-month-old infant has tested HIV-1 antibody positive.

Limitations of Routine Serological and Virological Testing of Newborns and Infants

First, passive in utero transfer of maternal HIV-1 immunoglobulin G (IgG) antibody has prevented the accurate and early serological diagnosis of HIV-1 infection in newborns. The mean duration of anti-HIV-1 antibody in these infants is

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usually 10 months (5, 66). Using enzyme immunoassay techniques on serially twofold-diluted maternal plasma obtained at delivery from seropositive women, we have shown that maternal HIV-1 IgG titers at delivery are consistently very high; HIV-1-specific IgG end dilution titers were commonly 1:1,000 (91). High antibody titers and the long half-life of IgG antibody (about 28 days) explain the persistence of this maternally derived antibody in the infants born to seropositive women and significantly limit the use of serology in identifying HIV-1 infections in these infants before 15 months of age.

Second, the timing and mechanisms governing the perinatal transmission of HIV-1 have not been clearly elucidated. Even though previous studies on abortus material have shown that transplacental infection can occur as early as 8 weeks of gestation (62), transmission at the time of delivery is equally possible. In the latter instance, testing of newborns for infection may give false-negative results.

Last, impaired B-cell function commonly accompanies progression of HIV-1 disease in infants, often resulting in profound hypogammaglobulinemia (36). In these cases, serology for HIV-1 antibody may be falsely negative. Whether early in utero infection results in significant impairment of immune function, in some cases leading to defective or absent HIV-1 antibody synthesis, is not known. Nevertheless, the serological testing and follow-up of these infants may be rendered inaccurate.

Virological testing of newborns and infants for HIV-1 may have its limitations as well. For example, the success of virological testing of infants by culture or by detection of viral antigen or viral nucleic acid is contingent upon the quantity of viable virus, circulating antigen, or infected T cells or tissues that harbor HIV-1-specific DNA. This quantity has been described as "viral load." Symptomatic patients, for example, who presumably carry a high viral load, are more often positive by virus culture, HIV-1 antigen testing, and HIV-1 DNA testing by polymerase chain reaction (PCR) than are asymptomatic patients (9, 51, 52). Schnittman et al. have used quantitative PCR techniques to show that HIV-1-infected adults who rapidly progress to symptomatic disease have more circulating infected lymphocytes than those whose infection is stable (87).

Newborns and young infants with HIV-1 infections may have absent to low levels of circulating virus, especially when mother-to-child transmission occurred during delivery. In this instance, virological testing may yield false-negative results. In fact, serial testing of infants by using viral culture or PCR has shown that a higher proportion of HIV-1-infected infants have positive results with these tests after 6 months of age than before that age (11, 74, 85).

Early identification of infected infants is crucial so that early intervention can be instituted. Developing a specific and sensitive method for detection in the very early stages of infection in the infant may be hampered by several factors discussed in this section. The following sections describe these assays for the detection of HIV-1 in infants.

SEROLOGICAL METHODS FOR DETECTION OF HIV-1

EIA and the Immunoblot Assay for Anti-HIV-1 Antibody

Enzyme immunoassay (EIA) for HIV-1 antibody is the most common method of screening for HIV-1 infection in adults and in children over 15 months of age. The sensitivity and specificity of the assay have been reported to be 99.5 and 99.8%, respectively (18, 27). The assay was developed initially to identify blood or plasma from HIV-infected donors (50). Although several commercial kits are available, they are all based on the same principle.

Virus is propagated in a chronically infected T-cell line (e.g., H9 cells) and isolated following cellular disruption with detergents. Purified viral proteins (HIV-1 antigens) isolated from the cell lysate are conjugated to a solid phase (e.g., polystyrene beads) (1). Patient serum or plasma specimens are incubated with the solid phase for up to 2 h. Antibody to HIV-1 in the patient sample binds to the antigen. The mixture is then washed free of any unbound material. Enzyme-linked anti-human IgG antibody conjugate is then added to the solid-phase antigen-antibody complex. After a second wash to remove excess unbound antibody, enzyme substrate is added, resulting in color development. The intensity of the color reaction correlates with the amount of anti-HIV-1 antibody in the sample and can be quantitated by using a spectrophotometer (27). Other EIA kits which use recombinant viral proteins or synthetic peptides or both instead of purified viral proteins have also been used for the detection of HIV-1 antibody.

Specimens that are repeatedly reactive on the EIA should be confirmed by the HIV-1 Western blot (immunoblot) assay. False-positive results on the EIA have been shown to occur in patients with a positive rapid plasma reagin test (37), hemophiliac patients (92), and patients on hemodialysis (77). Kuhnl et al. have also detected cross-reacting antibodies to HLA DR4 antigen (found in H9 cell lines) which seroreact when tested by EIA (56).

Immunoblotting (Western blotting) is used to confirm positive results on samples for anti-HIV-1 antibody. Partially purified virus, as described previously in the EIA method, is electrophoretically fractionated on polyacrylamide gels by molecular weight. The separated HIV-1 proteins are electrochemically transferred from the gel onto nitrocellulose membranes. Most commercial Western blot kits are supplied with nitrocellulose strips that contain the separated proteins. The test serum or plasma is then incubated with the strips. HIV-1 protein (or HIV-1 antigen)-specific antibodies in the sample bind to the membrane-bound antigen. Detection is achieved in situ with an enzyme-linked anti-human IgG antibody conjugate. The three major protein groups included in the assay are the Gag structural proteins (p55 and its protein subunits, p24 and p18), the envelope glycoproteins (gp160 and its subunits, gp120 and gp41), and the Pol protein enzymes (p66, p51, and p31). The CDC has recently established guidelines for interpreting immunoblot patterns and has recommended that samples be considered reactive by immunoblot when two or more bands for p24, gp41, and either gp120 or gp160 are present (19). Samples with fewer bands are indeterminate (19).

Approximately 20 to 30% of patients who are negative by HIV-1 EIA, as well as by the PCR test for HIV DNA, exhibit one or more reactive bands in an immunoblot test (27). These bands probably represent antibodies to non-HIV-1 antigens present in patient sera or plasma. The relatively high rate of nonspecific bands makes this test less useful as a screen for HIV-1 infection. The positive predictive value of the Western blot when used alone, especially when low-seroprevalence groups are tested, may be as low as 28.6% (27). However, the combination of the EIA and Western blot yields a positive predictive value of at least 99.5% (27). The present recommendation for HIV-1 antibody testing is to screen patients by EIA and confirm seroreactive samples by Western blotting.

Ten to 20% of sera reactive by EIA to HIV-1 are indeterminate by Western blotting (13). Celum et al. prospectively followed 89 individuals who were EIA positive but Western blot indeterminate to HIV-1 and reported a seroconversion risk of 4.5% in high-risk cases within a follow-up period of 10 months (13). The same investigators found a significantly higher risk of seroconversion among high-risk individuals when the original Western blot was positive for the p24 band. Patients who have indeterminate Western blot results should therefore be followed by serial testing for at least 6 months. Other adjuvant diagnostic assays, such as virological methods, may be helpful in these cases as well.

Passive in utero transfer of maternal anti-HIV-1 antibody to the fetus prevents the accurate serological diagnosis of HIV-1 in newborns and infants less than 15 months of age. Infants who remain seropositive after 15 months of age are considered infected with HIV-1 (17). Infants who initially test seronegative or who test seropositive but later lose antibody are considered uninfected, although there have been reports of EIA- and Western blot-negative infants who were positive for HIV-1 antigen, for virus by culture, or for HIV DNA by PCR (10, 35). Despite its limitations, longitudinal serological testing of infants born to seropositive mothers provides a fairly accurate, inexpensive, and universally available method for the definitive diagnosis of HIV-1 infections in this group of patients.

Occasionally, serial HIV-1 serological tests alone may be sufficient to diagnose HIV-1 infection in infants less than 15 months of age. For example, appearance of new bands or marked intensification of previously reactive bands on the immunoblot during follow-up testing of EIA-positive infants has been reported (5). This seroreactivity pattern strongly suggests endogenous production of infant IgG to HIV-1 antigens and hence is diagnostic of HIV-1 infection in these cases. In another case, an infant who seroreverted at 6 months of age became antibody positive again at 9 months of age, suggesting loss of maternally derived anti-HIV-1 antibody followed by endogenous production of the infant's own anti-HIV-1 antibody (5). Again, serological testing in this case was sufficient to diagnose HIV-1 infection.

Serological testing of newborns and infants for diagnosis of HIV-1 infection is hampered by (i) the seropositive status of virtually all newborns of infected mothers due to passive transfer of maternal anti-HIV-1 IgG antibody, (ii) the persistence of maternally derived antibody in the infant at a time when identification of infected infants is crucial, and (iii) the occurrence of HIV-1 infection in seronegative infants (10, 54, 82). Serial antibody testing by EIA and immunoblot, however, has been useful in eventually distinguishing uninfected infants (seroreverters) from those who are infected (persistence of antibody after 15 months or, less convincingly, appearance of new bands on immunoblot).

HIV-1-Specific IgG Subclasses, IgA, and IgM

Data regarding the course of anti-HIV-1 IgG antibody in infected pregnant women and their newborns are scarce. HIV-1-infected infants often manifest elevations in total immunoglobulins early in the course of the disease (36, 89). Eventually, significant impairment of B-cell function can lead to a state of severe hypogammaglobulinemia (36). Accordingly, symptomatic infants may lose their seroreactivity against HIV-1.

Pyun et al. have reported detection of HIV-1 infection in an infant by demonstrating production of IgG subclass-specific antibody against HIV-1 (80). The infant produced HIV-1 antibody of the IgG3 and IgG1 subclasses during the 5 months of observation, which suggests HIV-1 infection in the infant. Testing of HIV-1-specific IgG subclasses may therefore provide an acceptable method of detecting HIV-1 in newborns and infants; however, little information beyond Pyun's study is available.

Promising research has investigated the use of HIV-1-specific IgA as a diagnostic tool for HIV-1 in infants (58, 65, 81, 93, 97, 98). Testing for anti-HIV-1 IgA and IgM in infant sera provides an ideal method for detection of HIV-1 infection in infants because maternally derived IgA and IgM do not cross the placental barrier. Hence, the presence of anti-HIV-1 IgA or IgM in the infant should be diagnostic of infection. Previous attempts to develop an assay for detecting serum IgA and IgM have been hampered by the large amount of maternal IgG antibody in newborns that can competitively inhibit binding of serum IgA or IgM to test antigens (83). Weiblen et al. have shown that pretreatment of serum or plasma with protein G coupled to agarose beads significantly increases the sensitivity of the IgA or IgM assay (97, 98). When this technique is used, agarose beads are separated from the pretreated serum by centrifugation. Pretreated serum is then added to the solid phase coated with recombinant HIV-1 antigen (e.g., the protein gp160). Sera may also be incubated with partially purified HIV-1 proteins on nitrocellulose strips (standard immunoblot technique). After excess unbound antibody in the test serum is removed, enzyme-conjugated anti-human IgA is added to the mixture. The presence of anti-HIV-1 IgA antibody is confirmed by a color reaction step.

In the study by Weiblen et al., 42 (66%) of 64 samples from 38 infected infants demonstrated anti-HIV-1 IgA antibody by the immunoblot method after protein G pretreatment (97). Testing of sera against the gp160 recombinant antigen showed specific but less sensitive results (97). A later study by Martin et al. revealed similar results (65). Of 12 infected infants tested, 10 were positive for anti-HIV-1 IgA antibody (83% sensitivity, 100% specificity). Schupbach et al. reported the presence of anti-HIV-1 IgA in 48% of cord blood samples tested (88), but whether or not the relatively high perinatal infection rate evidenced by detection of anti-HIV-1 IgA in the cord blood could have been due to maternal contamination of the cord blood sample was not evaluated (88). A recent study by Quinn et al. showed an overall sensitivity and specificity for the anti-HIV-1 IgA assay for infants older than 3 months of age of 97.6 and 99.7%, respectively (81).

In summary, anti-HIV-1 IgA antibody testing in infants provides a specific and fairly sensitive method for detection of HIV in infants, but no conclusive information is available on this test in very young infants (less than 3 months of age).

Detection of anti-HIV-1 IgM has even less sensitivity for diagnosing HIV-1 infection in infants than detection of anti-HIV-1 IgA (83). The lack of sensitivity may be due to several factors: (i) as with detection of IgA, abundant anti-HIV-1 IgG may prevent accurate detection of IgM; (ii) IgM testing at birth or shortly thereafter may miss infants who were infected early in utero in whom the rise and fall of anti-HIV-1 IgM has already taken place; and (iii) among infants exposed during delivery, high maternal anti-HIV-1 IgG titers may blunt endogenous synthesis of HIV-1-specific IgM by inhibiting antigen recognition by B cells or HIV-1 infection itself may impair B-cell production of HIV-specific IgM (40). Johnson et al. serologically monitored 20 infants born to HIV-1-seropositive mothers and identified only 4 (50%) of 8 infected infants as anti-HIV-1 IgM positive (53).

In another study, only 4 (15%) of 27 seropositive infants (with indeterminate infection status) were anti-HIV-1 IgM positive, and none of the 3 infants less than 3 months of age were positive (40).

Although insensitive, tests for anti-HIV-1 IgM in infants have been shown to be specific. Pyun et al. reported the case of an infant with low levels of anti-HIV-1 IgM in cord blood who later demonstrated increasing levels of antibody that peaked when the infant was 8 weeks old, suggesting recent exposure to HIV-1 (80). Weiblen et al. have also demonstrated anti-HIV-1 IgM antibody in 21 (33%) of 64 samples from 38 infected infants (97). Interestingly, the same infants were also anti-HIV-1 IgA positive, but an additional 21 infants who were IgM negative were IgA positive.

Overall, present data on serum immunoglobulins in infants of seropositive mothers have shown that: (i) hypergammaglobulinemia (total IgG, IgA, and IgM) is a sensitive but relatively nonspecific early finding in HIV-1-infected infants; (ii) infected infants with advanced symptomatic disease may exhibit hypogammaglobulinemia presumably due to severe impairment of B-cell function; (iii) serial follow-up of anti-HIV IgG and possibly IgG subclasses is an inexpensive and acceptable method for diagnosing HIV-1 infection in infants, but the long duration of maternal antibody prevents early diagnosis; (iv) testing for anti-HIV-1 IgM or IgA is relatively specific but may not be adequately sensitive in young infants; (v) no definitive information on anti-HIV-1 IgA and IgM testing in infants less than 3 months of age exists; and (vi) pretreatment of infant serum or plasma with protein G to remove IgG may improve the sensitivity of IgA and IgM testing.

In Vitro Antibody Production and the ELISPOT Assay

The technique of in vitro antibody production is based on the principle that B cells in HIV-1-infected patients are usually activated in vivo and hence spontaneously secrete anti-HIV-1 antibody (73). Peripheral blood lymphocytes from infected individuals may be isolated and cultured in vitro and activated by mitogen such as pokeweed. In vitro antibody production involves coculture of mitogen-stimulated patient peripheral blood mononuclear cells (PBMCs) with normal healthy donor lymphocytes. The presence of HIV-1 is confirmed by periodically testing the culture supernatant for the presence of anti-HIV-1 antibody (29, 73, 95). Using this technique, DeRossi et al. identified 92.5% of 27 infected infants (29). Pahwa et al. reported similar success in detecting HIV-1 infection in both symptomatic (class P-2) and asymptomatic (class P-0) infants under 15 months of age (73). In the latter study, Epstein-Barr virus had been added to the cocultures, instead of mitogen, to provide the polyclonal B-cell activation that results in antibody production.

The ELISPOT test involves incubating antibody-producing patient PBMCs in polystyrene wells that have been coated with HIV-1 antigen. In theory, exposure of the patient PBMCs to antigen results in production of detectable anti-HIV-1 antibody. After the sample has been incubated and excess unbound antibody has been washed out, enzyme-linked anti-human IgG is added, and the presence of anti-HIV-1 antibody is confirmed by detection of "spots" at the bottom of the well, where the enzyme substrate had reacted. Using this technique, Lee et al. have demonstrated the presence of antibody-producing PBMCs in 27% of 30 infants born to seropositive mothers (60). In the same study, polyclonal B-cell activation was not observed when the PBMCs were exposed to tetanus toxoid, suggesting HIV-1 antigen-specific activation. No data regarding the sensitivity and specificity of the ELISPOT assay in very young pediatric patients exist, especially for infants under 3 months of age.

VIROLOGICAL METHODS FOR DETECTION OF HIV-1

Viral Culture Methods

Barre-Sinousi and coworkers successfully isolated HIV-1 in 1983 (6). Gallo et al. successfully cultured HIV-1 in 1984 by using H9 cells from a neoplastic T-cell line (HT) (41). Since that time, virus isolation from the cellular component of blood (48), plasma (26), body fluids such as vaginal fluid (79), amniotic fluid (70), and tissue (62, 64) has been reported. Recent advances have refined viral isolation techniques, greatly improving their sensitivity and specificity.

Patient PBMCs are harvested from heparinized blood by Ficoll-Hypaque gradient separation. The PBMCs are cocultivated with healthy donor mitogen-stimulated PBMCs in culture medium containing RPMI 1640, penicillin, streptomycin, 20% fetal calf serum, and interleukin-2 at 37°C in a 95% air–5% CO_2 environment. The cocultures are maintained by the periodic addition of interleukin-2 and fresh mitogen-stimulated healthy donor PBMCs. Culture supernatant is periodically collected (about every 7 days) and tested for either HIV p24 antigen or reverse transcriptase. Cultured cells may also be examined by light microscopy for syncytium formation, although this method may not be as specific as testing for viral products (5). Cultures that have not demonstrated evidence of HIV-1 infection by 42 days are usually terminated. HIV-1 has also been successfully cultured in non-T-cell-derived systems (e.g., in monocytes and macrophages) (49).

In early reports, the recovery rate of virus by culture of seropositive patients ranged from 70 to 90% (27, 61). Using a culture method similar to the one described above, Jackson and Balfour reported successful isolation of the virus in 97% (32 of 33 cases) of asymptomatic seropositive homosexuals and 100% (33 of 33 cases) of AIDS patients (51). Data on recovery rates of virus from infants less than 3 months of age are scant. One recent study estimated the percentage of culture-positive samples in infants tested very early in life (less than 3 months of age) to be approximately 50% (83, 85). Pahwa et al. recently showed that the poor sensitivity of viral culture in detecting HIV-1 improves when older infants are tested (74). In the latter study, the overall culture positivity rate among infected infants was 78% among all age groups, but the rate rose to 90 to 100% when samples were collected after infants reached 3 months of age (74). A similar study by Escaich et al. reported positive virus cultures in 8 (33%) of 24 infants born to seropositive mothers. Three of these eight infants were less than 1 month of age (34). We have recovered virus from infant blood as early as several hours after delivery of the infant (91). Of 12 infants born to seropositive mothers and sampled before 72 h of age, 3 were virus culture positive (91).

The poor sensitivity of virus culture to detect infection in the neonatal period (less than 1 month) may be due to several factors: (i) exposure to HIV-1 in these infants may occur during delivery, and testing for HIV-1 infection very early in life may therefore be falsely negative; and (ii) even if infection occurs in utero, the quantity of circulating cell-free or cell-associated virus in the infant may be below the level of detection by culture.

Successful recovery of virus by culture probably corre-

lates with the amount of circulating virus present in the infected patient or infant. Hence, the ability to culture virus from an individual may reflect this person's viral load. For example, Davey and Lane showed that 29% of culture-positive patients went on to develop AIDS within a 2-year period, compared with only 14% of culture-negative infected individuals (27). Time to positivity of the culture has likewise been shown to correlate with viral load; i.e., sample specimens from patients with a high circulating viral load are more likely to become culture positive in a shorter time (27, 83). Culture positivity and time to positivity of a given specimen may therefore be used as crude measures of viral burden.

Truly quantitative methods for viral culture have been reported by Ho et al. (48). In this study, patient PBMCs were 10-fold serially diluted and then cocultivated with a standard number of uninfected PBMCs. The highest dilution of PBMC that yielded a positive culture was considered the end point of the assay. The quantity of infectious HIV-1 was reported in tissue culture infective doses per number of PBMCs (48). In their study, asymptomatic but infected patients had 100-fold-lower tissue culture infective doses than patients with AIDS or AIDS-related complex. These data imply that asymptomatic patients have a lower viral burden than symptomatic ones. No definitive longitudinal data regarding the use of this quantitative method in newborns and infants are available. The application of such a method to neonates and infants with HIV-1 may provide some benefits (i) in identifying infected infants at risk for rapid progression to symptomatic disease, (ii) as a virological marker in monitoring the natural progression of HIV infection in infants, and (iii) in monitoring improvement among infected infants who are receiving antiretroviral therapy.

Other studies have reported recovery of virus in plasma and have investigated this method as a marker for progression of disease (26). Coombs et al. compared several possible markers of disease progression, including viral culture of PBMCs, viral culture of plasma, presence of HIV p24 antigen, and titer of antibody to p24 antigen (26). They concluded that plasma viremia, as demonstrated by isolation of virus in plasma, was a more accurate marker of progression of disease than either the presence of HIV-1 p24 antigen or antibody to p24. The role of this virological marker in infants and children as a diagnostic and, more importantly, prognostic tool is presently not known and requires investigation.

Nucleic Acid Hybridization and Amplification Techniques: PCR

Perhaps the most promising area of research for early diagnosis of HIV-1 infection in infants has involved detection of HIV-1 nucleic acid (DNA or RNA) sequences by gene amplification techniques, such as PCR. Detection of unamplified proviral HIV-1 DNA has been achieved by fluorescent in situ hybridization methods using U1 cells derived from a population of HIV-1-infected U937 cells (93). In situ hybridization techniques have also been applied to the detection of HIV-1 in PBMCs and lymph node biopsy samples from patients with AIDS or AIDS-related complex (46). Other techniques have been successful in the detection of either RNA or DNA genomic sequences and include dot blot (67) and Southern blot (90) hybridization techniques.

Increasing evidence, however, has shown that the circulating viral copy number in HIV-1-infected individuals may be as low as 10^3 DNA copies per ml of blood (44). Quantitative PCR studies have also shown that asymptomatic adult patients may have less than 1 infected T4 lymphocyte per 1,000 to 10,000 circulating T4 lymphocytes (87). Current nucleic acid hybridization probes are capable of detecting 10^4 to 10^5 DNA copies (44) and may therefore fail to detect DNA sequences in very low copy number, such as is possible in HIV-1 infections. Gene amplification, especially PCR, has revolutionized the application of nucleic acid hybridization techniques to the detection of proviral HIV-1 DNA sequences.

PCR was initially developed in 1985 (68, 69) and was first applied in the prenatal diagnosis of sickle cell anemia (32). Its application to the detection of HIV-1 followed shortly thereafter (52). The overwhelming interest in the development of this technique and its application to the detection of HIV-1 have resulted from several factors: (i) amplification of gene sequences with very low copy number markedly enhances the sensitivity of the nucleic acid probe assay; (ii) in the pathogenesis of HIV-1 infection, detection of HIV proviral DNA sequences represents an earlier marker of infection than detection of antibody; and (iii) a HIV-1-infected patient whose B-cell function is impaired may not be identified serologically but can be detected by this method. Use of amplification methods for diagnosis in newborns and young infants of HIV-1-seropositive mothers eliminates problems associated with antibody interference with serological assays. A brief description of the PCR techniques is given below.

The specimen usually tested by PCR is harvested PBMCs, although our laboratory has successfully detected proviral HIV-1 DNA sequences in maternal and infant urine, breast milk, and cervical secretions (unpublished data). Other studies have reported detection of HIV-1 DNA in plasma (47) and in fresh, formalin-fixed (57) or paraffin-embedded (43, 57) tissue samples.

With peripheral blood samples, PBMCs are obtained by Ficoll-Hypaque separation. The PBMCs are then lysed in a reaction tube containing proteinase K (38). The DNA from this digestion step is extracted with a mixture of phenol and chloroform. Either the resultant extract or the crude cell lysate before phenol-chloroform extraction may be used for PCR.

An aliquot of the cell lysate (or extract) is added to a reaction mixture containing a pair of oligonucleotide primers that are complementary to a defined segment of the HIV-1 genome. (Primers complementary to the long terminal repeat, *env*, *gag*, and *pol* regions of the HIV genome are available.) To this reaction mixture is added an excess of nucleotides required for DNA synthesis, magnesium sulfate, and a thermally stable DNA polymerase (usually obtained from the bacterium *Thermus aquaticus*). Amplification is carried out by using a DNA thermal cycler. Double-stranded DNA is denatured at 90 to 95°C. The temperature is lowered to 40 to 60°C to permit annealing between the oligonucleotide primers and sample DNA. Synthesis to double-stranded DNA is then achieved by raising the temperature to 70 to 75°C. The sequence of denaturation, annealing, and extension is then repeated for 25 to 40 cycles in the automated thermal cycler. The resulting PCR product is expected to contain x number of genomic copies, where $x = a(1 + e)^n$ and a is the original number of gene copies in the unamplified sample, e is the efficiency factor, and n is the number of PCR cycles for which the sample was run. An optimized PCR run would have an efficiency factor, e, close to 1. Optimization depends on several parameters, including presence of PCR

inhibitors (hemoglobin and heparin), concentration of magnesium, and actual temperatures chosen for each cycle.

Detection of amplified PCR product is usually achieved by hybridization with an oligonucleotide probe complementary to the original segment of HIV-1 genome that had been amplified. The probe is commonly radiolabeled with ^{32}P or ^{35}S, and the DNA probe-target complex is detected by autoradiography. Biotinylated probes have also been used effectively by some investigators (9), although nonisotopic detection appears to be 1,000-fold less sensitive than radioactive probes (44, 71). More recent data, however, on the use of biotinylated probes in detection of HIV-1 have been promising (11).

The sensitivity and specificity of PCR for HIV DNA have been reported to be approximately 97 and 100%, respectively (52), when HIV-1-infected adults are tested. Significant advances in the application of PCR techniques to the diagnosis of HIV-1 infection in infants and children have been made (24, 29, 30, 59, 76, 84, 96, 99, 100). DeRossi et al. compared various virological detection methods in the diagnosis of HIV-1 infection in infants, including HIV-1 antigen detection, virus culture, PCR, and in vitro antibody production (29). Of 27 infected children tested by these techniques in the first 6 months of life, 92.5% were positive by in vitro antibody production, 81.5% were positive by PCR, and only 70.3% were positive by culture (29). Antigen detection was the least sensitive method for diagnosing HIV-1 in the study group. Rogers et al. have also reported PCR-positive samples obtained during the neonatal period from five of seven infants who later developed AIDS and from one of eight infants who later had nonspecific signs and symptoms of HIV-1 infection (84). Overall, the composite data on PCR as a diagnostic assay for neonatal and pediatric HIV-1 infection have shown that (i) specificity is better than sensitivity when infants of any age are tested, (ii) sensitivity improves over time and probably approximates 100% when infants older than 6 months of age are tested, and (iii) data about the usefulness of PCR in infants under 3 months of age are scarce.

Less information is available concerning testing for HIV-1 RNA by PCR in infants and children. Briefly, RNA PCR involves synthesis of cDNA from native RNA by the addition of reverse transcriptase and nucleotides to the PCR reaction mixture. The PCR reaction is then initiated only after synthesis of cDNA has been accomplished, and the reverse transcriptase is inactivated by heating. Using this technique, Escaich et al. tested 12 newborns within 2 weeks after birth and identified 4 infants (33%) who were HIV-1 RNA positive, compared with 7 (58%) who were HIV DNA positive by PCR (34). Although less sensitive than DNA PCR, RNA PCR results were more predictive of which infants rapidly progressed to clinical disease. In Escaich's study, three of the four HIV-1 RNA-positive newborns became symptomatic after a mean follow-up of 6 months, while none of the DNA-positive but RNA-negative newborns became clinically symptomatic in the same mean follow-up period. This study suggests that HIV RNA positivity by PCR implies active viral replication and that such a state of replication may indicate a poor prognosis for the natural progression of HIV disease in infants (34).

Finally, growing research into the use of PCR in samples other than PBMCs (e.g., lymphoid tissue) is beginning to shed more light on the pathogenesis of HIV infection. Graziosi et al. performed quantitative HIV DNA PCR on PBMC and lymphoid tissue samples, obtained by biopsy, from both asymptomatic and symptomatic HIV-1-infected adults (42). In the study, a higher frequency of infected T4 lymphocytes was consistently found in lymphoid tissue than in PBMCs in all patients studied, suggesting that lymphoid tissue may be acting as a more efficient reservoir of infected cells than peripheral lymphocytes. No data comparing frequency of infection of lymphoid tissue and PBMCs in the neonatal and pediatric populations have been published. Data from such a study would provide much needed information about the role of other sites, such as lymphoid tissue, in HIV-1 disease in infants.

HIV-1 p24 Antigen Detection

Detection of viral fragments, specifically of the viral core protein p24, in serum or plasma has also been considered evidence of viral infection. Briefly, the assay for HIV-1 p24 antigen is a two-step "sandwich" technique in which human antibody to p24 is bound to a solid phase (either polystyrene beads or the bottom of a microtiter well) (2). Patient serum or plasma, which contains p24 antigen, is then incubated with the antibody. After excess unbound material is removed, anti-HIV-1 antibody (Ab-1) is added to the microtiter well or beads. After a second washing, enzyme-linked anti-IgG (Ab-2) is added to the sample. The final complex consists of the solid-phase p24 antigen, Ab-1, Ab-2, and enzyme (e.g., horseradish peroxidase). If viral fragments are present, an enzyme-dependent colorimetric reaction that can be detected and quantitated with a spectrophotometer occurs.

Using this technique, Borkowsky et al. found that 32 (86%) of 37 infected infants with AIDS had detectable antigen (9, 10). However, only 40 (68%) of 59 infants with AIDS-related illnesses and only 10 (37%) of 27 asymptomatic but infected infants were antigen positive (9, 10). In addition, none of the 10 specimens from HIV-1-infected newborns had detectable antigen. Use of the antigen assay in adult populations has shown the same trend, i.e., an overall poor sensitivity for detecting HIV-1 infection and more frequent positive results among symptomatic patients than among asymptomatic patients (3, 27).

Antigen positivity has been shown to correlate with rapid progression to symptomatic disease (33). AIDS and AIDS-related complex are commonly accompanied by active viral replication during which synthesis of viral product (p24 antigen or HIV-1 RNA) is active, whole virus in PBMCs (cell associated) or plasma (cell free) is easily recoverable by culture, and a greater proportion of T4 cells in blood or lymphoid tissue is infected with the virus. Hence, antigen positivity may be more useful as a prognostic marker for progression of disease than as a specific diagnostic tool for HIV-1.

Studies that compare antigen detection with virus culture and PCR have shown that the antigen test is least sensitive in the diagnosis of HIV-1 in infants (28, 29, 55). Krivine et al. reported a 96% concordance rate between viral culture and PCR results in PBMCs from 45 children born to seropositive mothers (55). However, only 6 (50%) of 12 virus culture-positive infants showed detectable antigen, while only 8 (44%) of 18 PCR-positive infants had antigen. DeRossi et al. have reported similar findings (28). In addition, the latter investigators have shown that false-positive results with the antigen assay are possible when infants are tested in the first 2 months of life.

Recent studies have shown that detection of HIV-1 p24 antigen may be enhanced by pretreatment of samples with acid (8, 72, 75). This is achieved by incubating serum

samples with hydrochloric acid (HCl) to a pH of 2.5 to 3.0. Acid pretreatment dissociates antibody-antigen complexes and therefore makes the antigen available to react in the EIA which follows. Nishanian et al. tested 652 antibody-positive serum samples by this method and showed that antigen positivity with acid pretreatment (50.6% of the sample) was significantly higher than that with no pretreatment (12.4%) (72). Preliminary studies by Bollinger et al. (8) and by our laboratory (91) have demonstrated promising results with the use of a glycine buffer (pH 1.8) for pretreatment of serum. The advantages of this method over the HCl pretreatment have yet to be proved.

Other Nonvirological, Nonserological Tests for HIV-1

No prenatal tests are available for detecting HIV-1. Immunohistochemistry and in situ hybridization methods that detect viral antigens in abortus material from HIV-1-infected pregnant women have shown evidence of HIV-1 infection in fetuses as early as 8 weeks (62). Culture and in situ hybridization have also confirmed the presence of HIV-1 in fetal organs such as thymus, spleen, brain, lung, liver, and placenta (63, 64).

Samples of chorionic villi, amniotic fluid, or fetal blood obtained from pregnant women by chorionic villus sampling, amniocentesis, or cordocentesis for obstetrical reasons could possibly be tested for HIV-1 by the methods described above, but studies in this area have yet to be initiated. Some studies, however, have reported the isolation of virus and the detection of HIV-1-specific IgM and IgA in amniotic fluid (12, 70). Successful isolation of virus and detection of viral fragments (nucleic acid or antigen) or HIV-1-specific IgA or IgM in these samples may provide a valid method of prenatal diagnosis of HIV-1. However, considerable attention should be given to the theoretical risk of infecting a fetus by using prenatal diagnostic procedures such as amniocentesis and chorionic villus sampling. HIV-infected pregnant women should be counseled about this risk when such procedures are offered.

Detection of HIV-1 in placental tissue may serve as a useful method for the diagnosis of HIV-1 in newborns for the simple reason that the placenta, which consists of trophoblastic tissue, is fetal in origin. Preliminary studies by Peuchmar et al. have shown that neither HIV proteins by immunohistochemistry nor HIV nucleic acid sequences by in situ hybridization were detected in 30 term placentas from seropositive mothers (78). In fact, 9 of the 30 infants whose placentas were studied were later proven to be infected with HIV-1.

β_2-Microglobulin and neopterin are by-products of increased mononuclear cellular activation and are therefore elevated during certain stages of HIV-1 infection (4, 25, 27). Both β_2-microglobulin and neopterin have been shown to correlate with the clinical stage of HIV-1 infection in adults, with significantly elevated levels among patients with AIDS (27, 39). Chan et al. compared levels of both of these markers in sera of symptomatic HIV-1-infected pediatric patients (CDC class P-1 and P-2) and noninfected patients (25). The study found significantly higher levels in both markers among the infected group. However, β_2-microglobulin and neopterin levels were not helpful in differentiating asymptomatic but infected infants (CDC class P-0) from noninfected patients. Interest in the use of the assays (usually EIA) that measure β_2-microglobulin and neopterin has somewhat waned because of their relative lack of specificity. In cases of neonatal and pediatric HIV-1 infection, measurements of β_2-microglobulin and neopterin may be more useful as prognostic markers for progression of disease.

SUMMARY

Cumulative data on serological testing of newborns and infants have shown that (i) maternal and newborn anti-HIV-1 IgG titers are high at delivery, which may explain the persistence of antibody in the infants of seropositive mothers; (ii) in some situations, serial HIV-1 antibody testing may identify infected infants; and (iii) detection of anti-HIV-1 IgA or IgM is specific for infection but the sensitivity of this assay may be compromised in certain situations, such as when infected infants are hypogammaglobulinemic or when the rise and fall of HIV-1-specific IgM synthesis following acute infection has been completed before delivery of the infant.

Cumulative data on PCR, viral culture, and tests for antigen in newborns and infants have shown that (i) among all age groups, viral culture is probably the most specific test available for detection of HIV-1, as PCR and the p24 antigen test may (though rarely) give false-positive results; (ii) the sensitivity of these tests increases in the order of antigen, culture, and PCR, with relatively insensitive results in the first 3 months of life for all of these tests; (iii) the sensitivity of all of these tests improves and approximates 90 to 100% when infants over 6 months of age are tested; and (iv) data regarding the sensitivity, specificity, and usefulness of these virological assays in infants under 3 months of age are very scant and inconclusive.

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