EXHIBIT E

Detection of Human Immunodeficiency Viruses by the Polymerase Chain Reaction

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• The human immunodeficiency viruses types 1 and 2 have been implicated as the etiologic agent of the acquired immunodeficiency syndrome and its related disorders. The direct detection of human immunodeficiency virus is complicated by the low incidence of free circulating virus as well as the small number of infected cells. An in vitro DNA amplification procedure known as the polymerase chain reaction has been applied to the detection of the human immunodeficiency virus proviral sequences in infected individuals. This article highlights the features of the polymerase chain reaction and its contribution to the detection of these viruses.

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Two human retroviruses of the Lentivirinae subfamily have been associated with acquired immunodeficiency syndrome. The viruses, human immunodeficiency viruses (HIV) types 1[1,2] and 2[3,4] share approximately 60% overall nucleic acid sequence homology for the more conserved *gag* and *pol* genes and from 30% to 40% for the other viral genes and long terminal repeat.[3] Human immunodeficiency virus 1 is the recognized agent of acquired immunodeficiency syndrome in the United States, Europe, and Central Africa, while HIV-2 is prevalent primarily in West Africa. The preliminary suggestion that HIV-2 is less pathogenic[5] merits further study.

The HIV life cycle[6] begins by attachment of virus particles to cells via CD4 receptors present most notably on mononuclear cells of T4 and macrophage lineages. On entry into the cell, the virus uncoats, and a DNA copy of the RNA genome (provirus) is synthesized by the viral encoded reverse transcriptase. The DNA can either circularize and integrate into the host genome or remain unintegrated in the cytoplasm. Human immunodeficiency virus replication is restricted at this stage until the infected cell is activated. The in vivo activation of HIV replication is not clearly understood, but in vitro activation can be accomplished by various agents, including interleukin 2[2] 5-iodo-2, deoxyuridine,[7] hydrocortisones and other hormones,[8] and tumor necrosis factor.[9] Spliced messenger RNAs encode multiple proteins involved in translational and transcriptional regulation of various stages of the viral life cycle; unspliced messenger RNAs encode the structural proteins of the virus. Posttranslational processing of viral structural polyproteins and assembly with genomic RNA at the cell surface results in the budding of mature virions.

The detection of antibodies to the viral proteins has played a significant role in the identification of individuals who are infected with these viruses. However, in many situations a direct detection of the virus would be desirable. Direct detection of HIV has proved refractory because of the small number of cells harboring HIV, the small number of proviral copies per infected cell, and the transcriptional dormancy of the viral genome.

Several procedures have been developed for detection of HIV. Of these, virus culturing has been the standard. However, even with cocultivation the successful isolation of virus from seropositive individuals and those with documented infection varies widely.[10] Moreover, the procedures are labor intensive, time-consuming, and costly. The use of in situ hybridization to directly detect viral nucleic acid in transcriptionally active cells[11] and Southern blot analysis of HIV DNA[12] extracted from lymphocytes has only been successful from a fraction of infected individuals. On the other hand, DNA isolation from lymph node and brain[13] has led to a higher, but still inadequate, proportion of individuals positive for HIV sequences by these procedures. The identification of viral p24 nucleocapsid antigens by specific polyclonal or monoclonal antibodies also suffers from the lack of sensitivity.[14] The sequestering of viral antigen in circulating immune complexes compromises this approach. Further-

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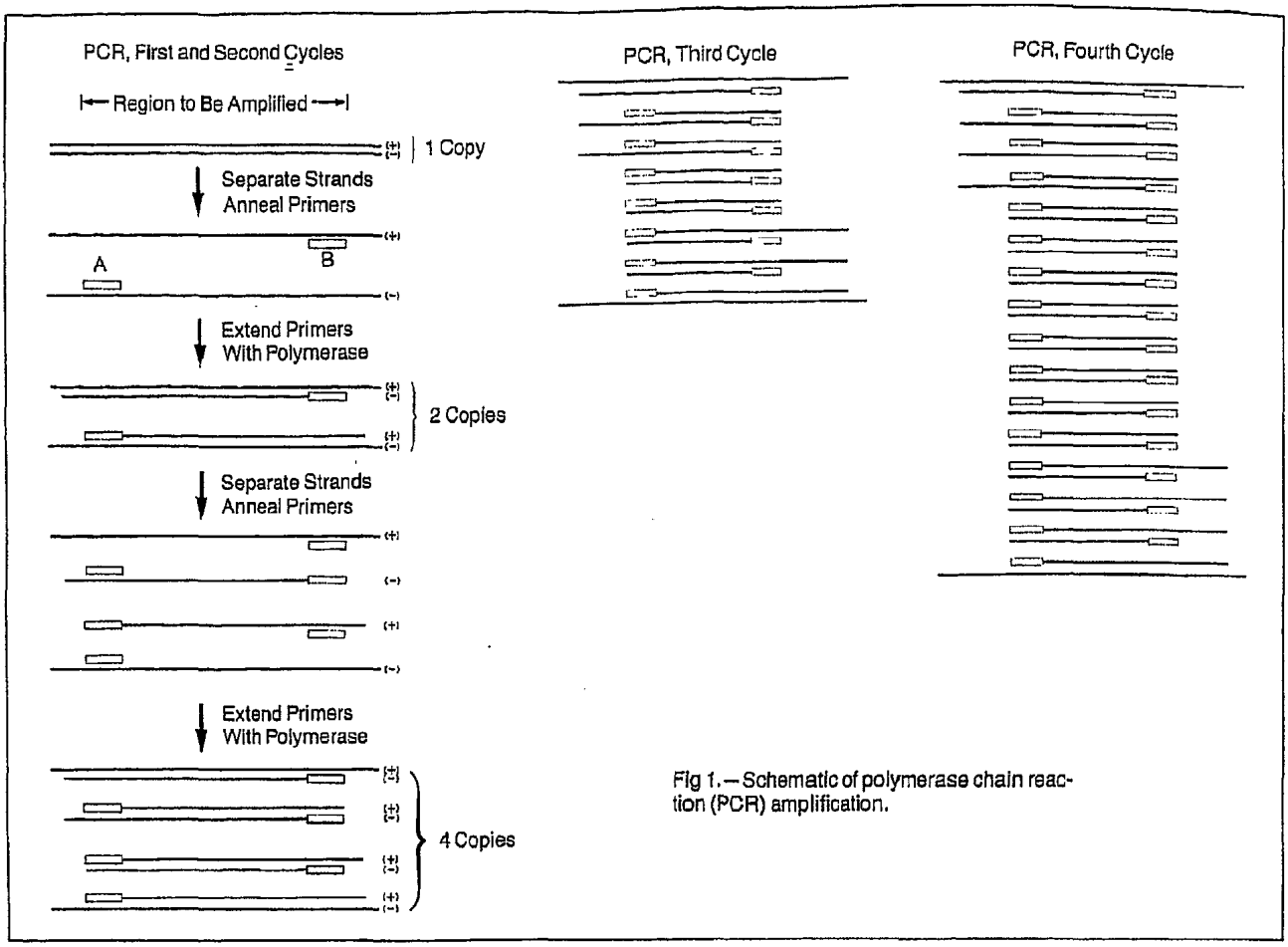

Fig 1.—Schematic of polymerase chain reaction (PCR) amplification.

more, antigens may not be detected during the latent stage when virus has established infection, but is not actively replicating.

An in vitro DNA amplification procedure, termed the *polymerase chain reaction* (PCR),[15,16] (Fig 1) has been successfully applied to the sensitive and specific detection of HIV proviral DNA. The use of a thermostable polymerase has further enhanced the application of PCR.[17] The procedure uses two synthetic oligonucleotides (primers) that are complementary to sequences that flank a region of interest; the upstream primer is complementary to the negative strand DNA and the downstream primer is complementary to the positive strand. Each cycle of PCR consists of a three-step process: denaturation of the DNA duplex, annealing of the primers, and extension of the primers with a polymerase in the presence of deoxynucleoside triphosphates. Theoretically, each cycle of PCR results in a doubling of the targeted sequence so that, for example, after 20 cycles of amplification a million copies of the DNA fragment are generated from a single copy. The procedure has also been applied to RNA by first transcribing the RNA to complementary DNA with reverse transcriptase and then amplifying the complementary DNA by the standard procedure.

The amplification of proviral DNA enables detection of not only cells actively replicating virus, but also those that harbor quiescent provirus. Although active HIV transcription can potentially generate a large number of transcripts, the limited number of studies exploiting RNA as a template[18,19] to date, suggest that more sensitive detection can be obtained if DNA is used directly as a template for PCR.[19] A productive area of research is the use of PCR to monitor and compare the messenger RNAs from the structural and regulatory genes.

Sequence variation will affect the stability of the primer-template duplex and, consequently, extension by the polymerase. Given the extensive heterogeneity of the viral genome,[12,20-23] it was necessary to amplify only highly conserved regions so as to provide detection of all viral variants both within and between infected individuals. The availability of nucleic acid alignments of all sequenced HIV isolates (Human Retroviruses and AIDS, Los Alamos [NM] National Laboratory, 1989) expedited the identification of conserved regions.

The commonly used primer-probe systems and reaction conditions for HIV amplification and detection have been described.[24,25] It is important to note that the reaction parameters must be optimized for each primer-pair system. The concentration of magnesium chloride, deoxynucleoside triphosphates, Taq DNA polymerase, and temperature-cycling profiles can all affect the specificity and, consequently, the efficiency of the amplifications. For HIV, we have designed primers that are specific for either HIV-1 or -2 as well

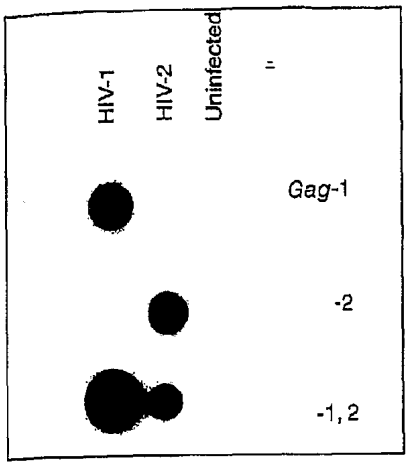

Fig 2.—Dot blot analysis of DNA extracted from individuals infected with human immunodeficiency virus (HIV) 1 or 2 that were amplified either with primers that are specific for both HIV-1 and -2 or with primers that are specific for each virus.

as primers that detect both viruses (Fig 2). For specific amplification of either HIV-1 or -2, regions with the least homology between the two viruses were targeted. On the other hand, only regions conserved between the two viruses were selected for simultaneous amplification.

Although the presence of large numbers (ie, 10 000) of HIV molecules in the original sample will result in amplification products that can be visualized on agarose gels following ethidium bromide staining, often the numbers of copies of HIV proviral DNA in a sample are small and may be as few as one copy in 10 000 cells.[11] The presence of small numbers of HIV molecules in a background of highly complex genomic DNA often results in the amplification of nonspecific products. Because the nonspecific products may be of the same length as the expected fragment, confirmation of the correct product is necessary and can be achieved through the use of a labeled probe that is complementary and between the primers of one strand of the amplified product. In addition to providing an extra level of specificity, the use of phosphorus 32–labeled probes provides the sensitivity often required for detection of products of low-copy number samples. When PCR is coupled with a detection method known as oligomer hybridization,[26] as few as 18 molecules of HIV in a background of 150 000 peripheral blood mononuclear cells or 1 μg of DNA can be detected.[27] Although purified DNAs were initially used, crude DNAs prepared by lysis of whole cells with proteinase K and nonionic detergents are now being used (Higuchi R., unpublished data, 1988, and reference 26). The use of Ficoll-Hypaque fractionated cells provides for the detection of HIV in not only $CD4^+$ cells, but in all mononuclear cells.

When PCR is used for detection of low copy targets such as HIV, extreme care must be taken to avoid cross-contamination of samples or carry over of amplified products that can lead to false-positive results. Guidelines for avoiding false-positive results with PCR have been described.[28,29] These guidelines include the using of positive displacement pipettes, the preparing and aliquot partitioning of reagents in an area that is free of PCR-amplified products, the sterilizing of reagents, and the frequent changing of gloves. Careful choice of positive and negative controls is important. Specifically, several "no DNA" reagent controls and negative sample controls should be included with each set of amplifications. The positive control should have a small number of target molecules so as to avoid the unnecessary generation of a large number of amplified targets. In addition, a small number of target molecules will also enable a better assessment of the efficiency of an amplification. A strict adherence to these guidelines has resulted in a dramatic reduction in the frequency and amount of carry over.

Polymerase chain reaction has been used for the direct detection of HIV-1 proviral DNA sequences in peripheral blood mononuclear cells of infected individuals, and has demonstrated clinical utility in several areas of diagnostics. For example, because of the window between infection and seroconversion, antibodies may not be detected in an infected individual. Although this seronegative window is typically 6 weeks to 6 months, protracted periods without production of antibodies have been described.[30,31] The PCR procedure detected the presence of HIV in high-risk individuals at least 6 months prior to seroconversion. A small number of individuals, initially identified as culture positive, demonstrated signs of infection by PCR for as long as 24 to 39 months prior to seroconversion. The observations noted here merit replication and further analysis.[31]

In seropositive individuals, PCR has been used to confirm the presence of HIV infection in individuals who were virus culture and/or antigen[25,32] negative. This procedure is currently being used in reference laboratories where HIV-1 proviral sequences have been detected in greater than 97% of the antibody positive individuals (Pathology Institute, Berkeley, Calif, and Specialty Laboratories, Santa Monica, Calif, unpublished data, 1989).

The procedure has begun to contribute to the resolution of the infection status of individuals with indeterminate serologic findings. In a small study of 100 low-risk volunteer blood donors with Western blot indeterminate patterns, Jackson et al[33] found that no one harbored HIV proviral sequences.

Polymerase chain reaction has been successfully used as a prognostic marker for infected infants born to seropositive mothers.[34-36] The persistence of maternal antibodies in babies for up to 18 months makes diagnosis by serologic assays difficult. When PCR is used to screen babies, those who were positive by PCR were either clinically ill or later showed signs of infection. Infants negative by PCR were later found to be seronegative and have remained clinically healthy. While the number of infants studied is small, the data thus far suggest that PCR can play an important role in the early identification of infected infants.

Polymerase chain reaction was used to confirm the first case of HIV-2 in a West African undergoing treatment in the United States.[37] In addition, the procedure was used to confirm the first documented case of a West African who was coinfected with HIV-1 and -2.[38] Similarly, individuals who were coinfected with HIV and human T-cell lymphotropic virus I were identified by PCR.[39,40]

Polymerase chain reaction has proved to be a valuable tool in several areas of research. First, the procedure has been used to study genetic variability in HIV-1 isolates.[22,23] Conventional means of restriction fragment polymorphism, although informative, provides only a limited assessment of heterogeneity. With PCR, the population of vi-

ruses, both in vivo and in vitro, can be analyzed. The sequences obtained from such studies should reflect more accurately the virus sequences present in an individual, as it does not require cultivation that may result in strain selection. Second, the demonstration that DNA extracted from formalin-fixed, paraffin-embedded tissue can serve as a template for PCR[41] suggests that archival material can be used to further elucidate the epidemiology of the virus. Third, experiments on the quantitation of PCR product suggest that the procedure could be used for the monitoring of viral load in patients receiving therapeutic agents.[42]

In summary, PCR has played an important role in (1) detecting of HIV sequences in early infection (2) identifying HIV sequences in seropositive individuals whose cells were culture negative for HIV after cocultivation and/or whose serum samples were negative for viral antigens, (3) prognostic screening of babies born to seropositive mothers, (4) resolving the infection status of individuals with indeterminate Western blot patterns, and (5) the typing of the human retroviruses present. The development of more rapid and inexpensive colorimetric assays will make the test more amenable for large-scale screening. In addition, PCR is expected to contribute to the diagnosis and study of other retroviruses.[39,43]

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