LABORATORY MONITORING OF THE VIRAL LIFE CYCLE

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Introduction: This review was not intended to be a comprehensive review of either the life cycle of HIV 1 or of all of the available assays for measuring HIV 1 (the reader is referred to numerous texts on these topics). Rather, the purpose of this presentation was to: 1) introduce the other conference topics in relationship to the life cycle of the virus, 2) increase our understanding that not all measurements will give the same results, and 3) increase awareness that the question being asked should drive selection of the measurement tool.

Background: Human immunodeficiency virus type 1 (HIV 1) is a member of the family Retroviridae, genus Lentivirinae, and is distinct from the other retroviruses such as the human T-cell lymphotropic viruses-1 and 2. There are two types of human immunodeficiency viruses, type 1 and 2. These are serologically and molecularly distinct from one another. HIV 1 is a genetically diverse collection of viruses which have been further subdivided into subtypes (clades). Some of the subtypes have greater or lesser molecular and serologic similarity with each other for which they have been lumped into groups. Dr. McCutchan gives a more detailed discussion of genetic diversity later in this conference proceedings.

HIV 1 is an integrated RNA retrovirus. It exists as either cell-free virus or cell-associated virus. In general, there is a very high ratio of non-infectious virus to infectious virus (1). In the cell-associated form, the virus can be integrated and latent, or it can be activated. The structure of HIV is relatively complex. The virus contains two strands of RNA that contain 9200 base pairs each. The RNA strands are associated with P7 nucleocapsid protein which is in turn encapsulated by the P24 capsid protein. Inside this core capsid are three enzymes of importance: a protease, a reverse transcriptase, and an integrase. The outside of the virion is comprised of a matrix protein that surrounds the core, adjacent to which is a lipid bilayer that is derived from the host cell plasma membrane. Embedded in the lipid bilayer are glycosylated GP41 molecules that anchor the external GP120 molecules. Both of these glycoprotein moieties are important for attachment of the virus to host CD4+ co-receptor complexes.

HIV 1 infection is characterized by three stages. First is the acute infection stage during which virus proliferates. This is generally followed by an asymptomatic clinical phase during which the immune system contains the virus to a varying extent, and antibodies to viral proteins are expressed. Finally, there is an immunodeficiency stage of the disease which is characterized by declining CD4+ cell counts to <200 CD4+ cells/&L of blood, increased susceptibility to opportunistic infections, and an increase in level of virus. Early diagnosis is thoroughly explored elsewhere in this conference proceedings.

Sources of Virus: HIV 1 virus has been isolated from many sources. The primary source for HIV 1 detection and quantification has been peripheral blood. However, the virus has also been isolated from breast milk, saliva, cerebrospinal fluid, bronchial fluids, feces, gastrointestinal aspirates and other body fluids and tissues. Of importance is the fact that HIV is a sexually transmitted disease. Although it has

been very easy to isolate the virus from blood, isolation of the virus from the genital tract has generally been more difficult. However, it is important to address detection and quantification of HIV 1 from the genital tract in order to assess factors influencing transmission and prevention of transmission. A number of investigators have assessed the ability to detect HIV 1 RNA in genital tract specimens. Drs. Fiscus (2) and Coombs (3) and their co-workers have addressed the ability to detect and quantitate HIV 1 in semen. They, and others, have standardized the procedures and characterized assay reproducibility with respect to peripheral blood. Although there are non-specific inhibitors of nucleic acid detection in both semen and female genital tract secretions, these inhibitors can be removed by a silica acid extraction prior to amplification of the viral RNA. If this pre-extraction methodology was employed, the standard deviations of the assays for nucleic acid detection were very similar to these assays for virus in whole blood (Fiscus, personal communication, 3).

Virus isolation from the genital tract in women is more complicated, primarily due to chronological order of sampling and choice of sampling methodologies. In one study (4), the order of sampling was assessed for cervico-vaginal lavage (CVL) and vaginal swab. When the CVL was obtained before the swab, swabs were more likely to be positive for both culture and RNA than if they were obtained first. Conversely, when swabs were taken first, the CVL samples were more likely to be positive than if CVL was done first. Thus, although sampling from the blood compartment at one time point may not affect another point, sampling from the genital tract by one method may impact results obtained by a subsequent method. Similarly, the choice of sample methodology can impact on the results. For example, in the aforementioned study (4), there was no difference in culture positivity for either lavage or swab samples with either collection methodology. However, measurements of viral RNA were more likely to be positive and had quantitatively higher levels of RNA when swabs were used to obtain the sample rather than CVL. Thus, depending upon the assay being utilized, i.e. culture or RNA, the choice of collection instrument may be important.

Overview of the Viral Replication Cycle: During the replication cycle, there is attachment of the virus to the cell, introduction of the viral RNA nucleocapsid complex into the cell, and reverse transcription of the viral RNA into double-stranded DNA. Reverse transcription is followed by integration of linear viral DNA into the cell's genome (provirus). If the cell becomes activated, there is transcription and translation of the proviral DNA (provirus) to produce new virions proteins and RNA genomes. This is followed by viral protein assembly, budding of virions from the cell membrane, virion release and maturation outside the cell. The following sections review the replication cycle and the relevant assays that are utilized to measure each one of the steps in viral replication.

Assays that Monitor Viral Attachment: Attachment has both virus and host dependent steps. Drs. Landay and Mildvan discuss the host contribution to viral attachment later in the conference proceedings. This section will primarily focus on the virological components. However, it is important to remember that viral attachment is not solely dependent upon the virus, but is also dependent upon host factors, e.g. co-receptor status of the CD4+ cells, presence or absence of specific co-receptor mutations, etc. In general, one refers to M-tropic virus strains and T-tropic strains, i.e. strains that infect macrophages or strains that infect T-cells, and we group these strains as non-syncytium inducing (NSI) and syncytium inducing (SI) types of viruses, respectively. The dogma is that macrophage or NSI variants use CCR5 host co-receptors and SI or T-tropic viruses use host CXCR4 co-receptors. However, such a simple distinction disregards the gray zone in-between.

There are a number of attachment related measurements that have been developed. There are binding assays, blocking assays, and tropism assays. The initial attachment assays were based on cell fusion. The system used consisted of a receiving cell system that utilized a gene encoding for the E. coli gene for beta-galactosidase along with bacteriophage T7. Fusion of the T7 polymerase with the T7 promoter and the lac V gene results in expression of beta galactosidase, which, in the presence of substrate, produces a product that can be measured colorimetrically. This technique was used to define that T-trophic strains utilized the host CXCR4 receptor and M-trophic strains used the host CCR5 binding receptor (5). Another measure of tropism is the production of syncytium. A surrogate marker for syncytium production is replication in the MT2 cell lines. Strains that induced syncytia would replicate in MT2 cells, while strains that were NSI would not. From a technical standpoint, the MT2 assay has been standardized and validated (6). Furthermore, since most clinical isolates are mixtures, the MT2 assay has been shown to be capable of detecting one tissue infective dose 50 in a potential mixture of SI and NSI variants (6).

The above assays are qualitative in their assessment of viral attachment and replication. It is also possible to study the replication kinetics of virus strains in various cell types. A series of replication kinetics curves can be generated for the viruses in various host cells. This type of approach describes viruses that are neither "black or white" in terms of co-receptor usage. Dr. Lathey and co-workers have derived a definition of macrophage versus T-tropic cell virus based on replication kinetics (7). These investigators correlate replication kinetics of SI and NSI variants using monocyte-derived macrophage cultures with receptor usage using standardized biological clones of some of these SI and NSI isolates and some clinical isolates. These results have been compared with the co-receptor usage assays in terms of the SI and NSI phenotypes. Of 5 SI isolates, 2 used CXCR4, 2 used CCR5, and 1 used CCR5 and CCR3. Differences in replication kinetics were observed for viruses using solely CCR5 regardless of their SI phenotype. These data illustrate the complexity of basing an assignment to attachment and replicative category on one assay. This is one of many examples where the results from one assay may not be directly comparable to results from another assay.

Assay Measuring Viral Replication: Viral replication, like viral attachment, is both host and viral dependent. Drs. Michael, Landay and Mildvan discuss the host contribution in greater detail later in this conference proceedings. Down regulation of HIV replication is associated with numerous factors, including HIV-specific immune responses, CD8+ suppressor factors, beta-chemokines, and inhibitory cytokines. Up regulation of HIV replication is associated with cell activation, pro-inflammatory cytokines and with opportunistic infections. HIV replicates most efficiently in activated cells. Since the consequence of HIV infection is a high level of immune activation, a circular pattern develops whereby host response can result in increased viral replication.

Measuring the reverse transcription step. The reverse transcription (RT) enzyme is an RNA-dependent DNA polymerase. This enzyme is responsible for the synthesis of complementary viral DNA from the viral RNA, which is then integrated into the host cell DNA. There are many errors that can arise during the transcription process, which explains, in part, the considerable genetic diversity of this virus. It is not clear exactly at what time point following infection, or where in the cell RT activity occurs. The RT activity probably is initiated in the cytoplasm shortly after viral entry and continues inside the nucleus. There are many cellular factors that can influence RT activity. Early in the days of HIV research, measurement of RT activity was utilized as a measure of viral replication. However,

the great variation in results that accompanied varying culture conditions did not lend easily to RT standardization. An ultrasensitive RT assay has been developed that may have utility in screening for retroviruses of unknown viral-sequence (8).

Measuring pro-viral copy number. The viral DNA that is synthesized by RT can exist in many forms, including linear, circular, integrated and unintegrated. A more detailed discussion of pro-viral copy number determinations can be found later in the conference proceedings by Dr. Coombs. There are many methods for assessing viral DNA. The more elegant of these methodologies combines tissue-specific localization of viral RNA and DNA products, which identifies those cells that are either productively or latently infected (9, 10, 11). Early studies for quantifying pro-viral copy number were semi-quantitative, used radioactive detection methods and were not amenable to easy standardization. The newer methods utilizing internal standards or external standard curves with optical density readouts appear quite promising for use as standardized measurement tools. There is much more viral DNA made than is integrated into the cell nucleus as pro-virus. Various approaches have been taken to distinguish integrated from unintegrated viral DNA (see Dr. Coombs section, 11). Thus, as with attachment, not all assays of "pro-viral DNA" measure the same viral component.

Measuring latent, culturable virus. Assessment of integrated pro-viral DNA that is capable of producing virus is accomplished through cell culture. The culture techniques involve co-cultivation of peripheral blood mononuclear cells (PBMC's) obtained from HIV-infected patients with immune-stimulated PBMC's obtained from uninfected donors. Viral replication in culture is generally assessed by measuring the production of the viral-encoded p24 antigen. The p24 antigen assay will detect virus in infected cells from the host that are activated, as well as those that can be activated in vitro. These culture techniques have been standardized (13) and are able to detect virus in the majority of untreated patients (14). Recently, investigators have utilized culture systems that remove CD8+ cells prior to culturing the CD4+ cells to try to increase sensitivity of the standard culture assay (15,16). It is not clear how much this step adds to the sensitivity of standard culture methodologies. The major problem with culture methodology is that the method is semi-quantitative. Since the assay involves a series of dilutions, there will always be a dilutional variability associated with cell culture, which is not found in other linear assays such as RNA quantification.

Measuring products of transcription. A host cell polymerase initially transcribes a portion of the integrated viral DNA. The initial transcription/translation of viral DNA by host cell occurs at a very low level and is incomplete. The early-synthesized fragments of messenger RNA are referred to as, spliced messages. The messages are translated in the cytoplasm, with resultant formation of a number of viral regulatory proteins. These regulatory proteins migrate back into the nucleus and initiate synthesis of copies of viral RNA, termed unspliced messenger RNA, which is generally considered genomic RNA. Several assays systems have been developed to measure spliced and unspliced RNAs (17, 18).

The significance of these assays for RNA in predicting disease progression or for monitoring therapy is unclear. Some investigators have found a positive association with disease progression, and other reports in the literature have found none. As with many issues in HIV research, these results reflect differences in the technologic approach. In many of these studies there are differences in the primer pairs that are used to define unspliced, single spliced, and multiple spliced RNA (18). Thus, depending on the primer pairs, or the manner in which the assay was performed, these procedures are not

necessarily measuring and detecting the same component.

Measurements of translation. Viral messenger RNA is translated into the gag, pol and env gene products. One of the major translational products from the gag gene is the nucleocapsid antigen p24. The p24 antigen can be detected either intracellularly or extracellularly as an intact virion. There are multiple kits for measuring p24 antigen, which are inexpensive and technically simple. The major limitation of this assay has been lack of sensitivity, since only patients with very low CD4+ cell counts and high viral loads by other methods are likely to be p24 antigen positive. The use of methods that dissociate the p24 antigen from antigen-antibody complexes has increased the sensitivity of the assay, but generally not to the extent of RNA assays. Combining immune complex dissociation methods with a signal amplification boosted HIV 1 p24 antigen enzyme-linked immunoassay (ELISA) has led to p24 antigen detection in plasma that is as sensitive as RNA detection (19).

Virus Assembly and Maturation: The viral protease, which is a product of the pol gene, plays a key role in viral assembly and maturation. Post-translational processing of the viral gag and pol gene products by protease to give the structural and functional proteins for the mature virion probably occurs at the cell surface and/or during the budding process. This processing is necessary for production of virus that is infective. As the virus buds from the cell surface, both host-cell proteins and viral-envelope glycoporteins are incorporated into the virion. The newly released virion is in an immature form. Viral maturation occurs post-release from the host cell in the extracellular milieu. Electron micrographs of cells in which HIV 1 replication is occurring regularly depict numerous immature virions and few mature virions.

Measuring plasma virion RNA. There are numerous commercial kits now available for measuring plasma virion RNA. Dr. Yen-Leiberman gives a more complete discussion of plasma RNA determinations later in the conference proceedings. It is important to remember that the kits do not all measure viral RNA in the same way, thus there are intrinsic differences in the assays that can lead to different results. This is particularly the case for the genetically diverse group of retroviruses as Dr. McCutchan discusses later. Finally, it must be remembered that plasma virion RNA is not a measure of infectious plasma virus per se since most of the virions are not replicatively competent as discussed above

Measuring culturable plasma virus. The only true measure of cell-free infectious virus is obtained by measuring the culturable virus in plasma. Virus isolated from plasma represents active viral replication in vivo. However, patients do not usually become plasma viremic until very late in their disease (20). The methodologies for optimizing the assay have been published (21).

Viral Drug Resistance: Viral drug resistance can be measured for either cell-associated virus or cell-free virus. Cell-free virus is thought to be the strain immediately in circulation, while cell-associated virus is thought to be archival virus. Furthermore, viral resistance can be measured either phenotypically or genotypically (22). Finally, since the virus circulating at any point in time usually consists of a mixture of viruses, resistance can be measured either in aggregate pools of the viruses or in individually cloned viruses. There are almost as many assays for resistance as there are HIV variants. Thus, when one asks scientific questions regarding the relationship of viral resistance to disease progression, or how the knowledge regarding resistance helps in patient management, the answers are dependent on the measurement technology employed.

Dr. Schuurman will describe various resistance assays in greater detail in the conference proceedings, and will outline results obtained from initial quality assurance efforts for genotypic analysis. The early attempts at assuring quality of phenotypic assays were problematic since to perform the assay each laboratory had to pass the virus in cell culture, and thus, there was no guarantee that all isolates would be the same. A similar problem arose when attempting to quality assure the genotypic analysis. The viral isolate, although sub-cultured many times at a central laboratory, contained a mixture of wild-type and mutant virus. Some laboratories called the virus resistant and some not resistant. It was only after clonagenic analysis that it was recognized that the virus isolate contained a mixture of mutant and wild-type virus. The approach Dr. Schuurman will describe uses cloned plasmids. While using plasmid clones will aid in standardizing the various genotypic assays, it will not answer the question of which assays or approaches to use for measuring resistance in patient viral isolates.

Summary: In summary, there are numerous laboratory measurements for HIV. The measurements may reflect any of several stages in the life cycle. In the adage of the blind man and the elephant, each measurement offers a different perspective. Our understanding of the clinical management of HIV must come from a variety of different measurements. Most importantly questions being asked should define the measurement, and not that the measurement define the question.

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