CELLULAR ASPECTS OF HIV INFECTION

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CELLULAR ASPECTS OF HIV INFECTION
CYTOMETRIC CELLULAR ANALYSIS

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Infection with human immunodeficiency virus (HIV) has produced one of the most dramatic epidemics of the twentieth century. It has spread worldwide, leaving no region of the world unaffected. Before effective therapies were developed, infection with HIV meant an inexorable decline in health until death was a welcome relief. Now that the capability to decrease viral replication has been achieved, those who can afford this expensive treatment survive by keeping the infection dormant, not by eliminating the virus altogether. Unfortunately, the antiviral reagents available come with serious side effects, and resistance to these agents develops readily for a consistent percentage of patients. At the same time that therapies with specific antiviral agents have decreased morbidity and mortality, they have resulted in a relaxation of appropriate public and private health measures, which threatens a recrudescence of epidemic infection. Of course, many if not most infected persons worldwide cannot even afford treatment and perish quickly without any specific intervention.

Many biomedical scientists have investigated HIV and the disease syndrome that it produces in infected persons. These investigators have contributed greatly to our understanding of the mechanisms that the virus uses to replicate, to infect new hosts, and to cause disease. These mechanisms have been described in molecular, cellular, organismal, and social terms.

At the cellular level, investigators first identified the cells that are infected by HIV or that act as reservoirs for the virus. Then the crucial mechanisms of the immune response, including the importance of HIV-specific cytotoxic cells and humoral responses, the way in which cells die after the infection, the death of innocent bystanders, and the role of costimulatory molecules and coreceptors were described. These studies at the cellular level have relied on many different technologies, one of the most important being flow cytometry.

Flow cytometry is a powerful technique for the analysis of multiple parameters of single cells. It is capable of assessing six to ten parameters on 10,000 cells in less than a minute. Moreover, cells with specified characteristics can be sorted live and cultured for additional investigation. Flow cytometry has been used since the beginning of the HIV era as a key approach to study the cellular
level in HIV infection. Millions of analyses have been performed on samples from persons infected with HIV. These analyses have allowed us to follow the course of the infection, to observe the complex response of the immune system to the virus, and to help in deciding how to treat infected patients, and to understand the patients' cellular response to the therapy.

This book includes chapters by renowned experts on various aspects of HIV investigations. The main aim of this book is to present these descriptions and analyses with particular attention to the role that flow cytometric techniques have played in shaping our current conceptualizations. The book is divided into five parts—molecules, cells, pathophysiological processes, technologies, and organisms. Each chapter emphasizes an intelligent, concise synthesis of the topic without an attempt to provide an exhaustive review.

The book is intended for experts in the field of HIV studies, including immunologists, virologists, and clinicians, as well as for other researchers who are primarily interested in the use of flow cytometric techniques in biomedical investigations.

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PART I

MOLECULES
INTRODUCTION

The precise understanding of the molecular mechanisms in each step of the human immunodeficiency virus (HIV) life cycle has provided an essential basis for designing antiviral compounds and strategies aimed at blocking viral replication and preventing or delaying disease progression. As in other retroviral infections, the replication cycle of HIV can be described as proceeding in two phases. The first phase includes entry of the virion into the cell cytoplasm, synthesis of double-stranded DNA (provirus) using the single-stranded genome RNA as a template, transfer of the proviral DNA to the nucleus, and integration of the DNA into the host genome. The second phase includes synthesis of new copies of the viral genome, expression of viral genes, virion assembly by encapsidation of the genome by precursors of the HIV structural proteins, budding, and final processing of the viral proteins. Whereas the former phase is mediated by proteins that are present within the virion and occurs in the absence of viral gene expression, the latter, leading to production of infectious virions, is a complex process requiring the interplay of viral and cellular factors.

The precise understanding of the molecular mechanisms of HIV replication and the use of new technologies in virology has lead to exciting discoveries on many aspects of the biology of this virus. In particular, a growing body of new data on the HIV replication mechanisms together with the results from molec-
ular studies carried out directly in vivo have allowed researchers to address the virus-host relationships, including the pathogenic role of this virus in disease progression.

In this chapter, two virologic aspects that are regulated by the complex mechanisms of the HIV-host interplay and have crucial pathogenic implications are discussed: the dynamics of HIV replication and the intrahost evolution of the HIV population.

**HIV GENOME AND CONTROL OF VIRUS EXPRESSION**

The HIV genome encodes for precursor polypeptides of structural and functional virion proteins, regulatory proteins, and other proteins that are dispensable for replication and are called *accessory* proteins (Table 1.1). As for other

<table>
<thead>
<tr>
<th>HIV Gene</th>
<th>Protein</th>
<th>Function</th>
<th>Essential for Replication</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gag</em></td>
<td>Pr₅₅&lt;sup&gt;gag&lt;/sup&gt;</td>
<td>Polyprotein precursor for matrix protein (p17), capsid protein (p24), nucleocapsid protein p9, and p7</td>
<td>Yes</td>
</tr>
<tr>
<td><em>pol</em></td>
<td>Pr₁₆₀&lt;sup&gt;gag-pol&lt;/sup&gt;</td>
<td>Polyprotein precursor for virion enzymes reverse transcriptase (RT)/RNAse-H (p51), protease (PR) (p10), and integrase (IN) (p32)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>env</em></td>
<td>gp160</td>
<td>Polyprotein precursor for envelope glycoproteins gp120 and gp41 (receptor binding and membrane fusion, respectively)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>tat</em></td>
<td>p14</td>
<td>Transcriptional transactivator (initiation and elongation of viral transcripts)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>rev</em></td>
<td>p19</td>
<td>Regulates viral gene expression at post-transcriptional levels (regulates splicing and transport of viral RNAs from the nucleus to the cytoplasm)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>nef</em></td>
<td>p27</td>
<td>Downregulates CD4 receptor, enhances virion infectivity, influences T-cell activation</td>
<td>No</td>
</tr>
<tr>
<td><em>vif</em></td>
<td>p23</td>
<td>Viral infectivity factor (infectivity reduced in <em>vif</em>-minus mutants)</td>
<td>No</td>
</tr>
<tr>
<td><em>vpr</em></td>
<td>p15</td>
<td>Virion protein (associated with the nucleocapsid) implicated in regulation of viral and cellular gene expression</td>
<td>No</td>
</tr>
<tr>
<td><em>vpu</em></td>
<td>p16</td>
<td>Influences virus release</td>
<td>No</td>
</tr>
</tbody>
</table>
retroviruses, the genomic HIV RNA is synthesized and processed by the cellular mRNA handling machinery starting from the proviral HIV DNA. For this reason, the viral genome contains a cap structure at the 5' end and a poly-A tail at the 3' end. Moreover, the diploid lentiviral genome has the additional feature of being rich in A residues (on average 38–39%) (Myers and Pavlakis, 1992). As a direct consequence, the HIV codon usage differs dramatically from that of cellular genes (Berkhout and van Hemert, 1994; Kypr et al., 1989).

Control of HIV RNA synthesis is complex and requires the presence of several cellular proteins as well as of viral transactivators and cis-acting viral elements. Indeed, retroviral long terminal repeats (LTRs) are divided into domains (designated U3, R, and U5) that have distinct functions in transcription either in regulating basal levels or inducing high levels of HIV gene expression. The U3 domain of HIV contains basal promoter elements, including the TATA box for initiation by RNA polymerase II and the site for binding the cellular transcription factor SP1. Immediately upstream of the core promoter, the virus contains one or more copies of a 10-bp sequence recognized by the enhancer factor nuclear factor (NF)-κB. However, whereas in simple retroviruses regulation of viral transcription is passive (i.e., regulated by cellular factors), in HIV infection, this process is more complex and products of the HIV genome are required to achieve high levels of expression. Initiation of HIV RNA occurs at the U3/R level (cap site) of the 5' LTR, and the viral transactivator Tat functions through a cis-acting sequence (designated Tat-responsive element, TAR) an RNA encoded by a region located in R (+19 to +43). R-U5 is the leader sequence of the full-length and spliced viral transcript, whereas the 3' ends of mRNAs are defined by the R/U5 border in the 3' LTR. Finally, the accessory genes of HIV (vif, vpr, vpu, and nef) (Table 1.1) are generally defined as dispensable for viral replication based on studies in tissue culture systems. On the other hand, accessory genes are expressed in vivo and increasing data indicate that they play important roles in the virus-host interplay.

MOLECULAR CORRELATES AND DYNAMICS OF HIV ACTIVITY
IN VIVO

The relevant data on mechanisms of HIV replication have been coupled with the results from in vivo studies, thus obtaining a precise understanding of the virus-host relationships. Indeed, natural history and pathogenicity studies have supplied a profile of HIV activity during the different phases of this infection, have contributed to a better understanding of virus-host interactions, have allowed the application of mathematical models to evaluate the intrahost HIV dynamics, and, finally, have provided a theoretical basis for therapeutic antiviral intervention.

In vivo, systemic HIV activity is a formal entity that consists of a sum of dynamic processes, including productive infection of target cells, release of virions outside the infected cell and eventually in the blood compartment, and
de novo infection of permissive cells. The virus variables influencing the level of systemic HIV activity and cell-free virus dynamics include degree of viral expression and host cell range, whereas the host variables include the specific (humoral and cytotoxic) immune response and polymorphism of genes coding for cell receptors of HIV.

The vast majority of quantitative studies carried out in vivo have highlighted the role of cell-free viremia as a reliable index of mean viral activity in HIV infection. Indeed, viremia-based studies have provided clear evidence that changes in HIV load during the different phases of this infection can be efficiently evaluated by measuring cell-free virus in plasma samples (Bagnarelli et al., 1994; Perelson et al., 1996), and that substantial increases in viral load parallel or even predict (Mellors et al., 1996) the disease progression. These findings have greatly contributed in the last few years to a clearer understanding of the virologic correlates of disease progression, to driving new attempts at understanding the pathogenic potential of HIV, and to designing effective antiretroviral strategies. Although recent research has highlighted the diagnostic role of other quantitative parameters, including viral transcription pattern and provirus copy numbers, and although in some cases virus compartmentalization may influence the exact correspondence between cell-free plasma viremia and systemic viral activity, the analysis of viral genome molecules in plasma samples is still a major molecular correlate of systemic viral activity at the level of the whole body in many human viral infections.

The evaluation of patients undergoing potent antiviral treatments has allowed the dynamics of cell-free virus in plasma to be addressed in vivo (Ho et al., 1995; Perelson et al., 1996; Wei et al., 1995). Importantly, these studies have documented the dynamics of cell-free virions in plasma (half-life being approximately 5.7 h) and the turnover of infected cells. Furthermore, the sensitivity and specificity performances of most quantitative molecular methods have provided in the last few years a simple approach to the evaluation of gene transcription in vivo and in vitro. In HIV infection, consistent evidence has indicated that progression of disease is driven by an increase in viral load evaluated as cell-free plasma virus. To address whether this increase is contributed by the dysregulation of the molecular mechanisms governing virus gene expression at the transcriptional or post-transcriptional levels, several quantitative virologic parameters (including provirus transcriptional activity and splicing pattern) have been analyzed in subjects with nonprogressive HIV infection and compared with those of matching groups of progressor patients. It was observed not only that high levels of unspliced (US) and multiply spliced (MS) viral transcripts in peripheral blood mononuclear cells (PBMCs) correlate with the decrease in CD4+ T cells (Bagnarelli et al., 1996; Furtado et al., 1995) following the general trend of systemic HIV-1 activity, but also that MS mRNA levels in PBMCs are closely associated with the number of productively infected cells (Bagnarelli et al., 1996), because the half-life of this class of transcripts after administration of a potent protease inhibitor is very consistent with that of productively infected cells. The transcriptional pattern observed during in vitro
infections of T-cell lines, primary PBMCs, and monocytes/macrophages supports these findings.

### INTER- AND INTRASUBJECT HIV VARIABILITY

Comparative analysis of the sequences of the HIV env gene from a great number of viral isolates has revealed a pattern of five hypervariable regions (designated V1 to V5) interspersed with more conserved sequences in the gp120. This sequence variation consists of mutations (resulting in amino acid substitutions), insertions, and deletions (Leigh-Brown, 1991). Among HIV isolates from geographically different locations, gp120 amino acid sequences may diverge up to 20–25%, whereas other regions of the genome are relatively conserved. More recently, molecular epidemiology surveys based on env sequences of numerous HIV isolates have revealed at least nine distinct HIV subtypes (or clades) in the acquired immunodeficiency syndrome (AIDS) pandemic (Myers, 1994; Myers et al., 1994) (intersubject HIV variability).

Subsequent analysis has revealed that both linear and conformational determinants influence the functional and antigenic structure of the gp120; this is a crucial pathogenic issue, inasmuch as all neutralizing antibodies are directed against env-encoded domains in HIV-infected hosts. Indeed, infections with retroviruses are also characterized by different (from moderate to high) levels of intrahost viral genetic variation. This viral variability is dependent upon mutation, recombination, degree of viral replication, and the host’s selective pressure (Dougherty and Temin, 1988; Hu and Temin, 1990; Pathak and Temin, 1990a, b; Temin, 1993). In HIV infection, the viral population is represented by related, nonidentical genetic variants (Goodenow et al., 1989; Hahn et al., 1996; Meyerans et al., 1989; Pedroza Martins et al., 1992), designated quasispecies. The error-prone nature of the HIV reverse transcriptase (RT) and the absence of a 3′-exonuclease proofreading activity determine in vitro about $3 \times 10^{-5}$ mutations per nucleotide per replication cycle (Yu and Goodman, 1992). Although the mutation rate observed in vivo is lower than that predicted from the fidelity of purified RT (because a number of newly generated variants are unable to replicate or are cleared by the host’s immune system) (Mansky and Temin, 1995), the viral replication dynamics (Ho et al., 1995) and the host’s selective forces determine a continuous process of intrahost HIV evolution (Bagnarelli et al., 1999; Holmes et al., 1992; McNearney et al., 1992; Wolinsky et al., 1996). Overall, the data currently suggest that viral genetic variability is the molecular counterpart of a continuous dynamic interplay between viral (i.e., HIV-1 replication dynamics and generation of variants by mutation and recombination) and host factors (i.e., selective pressure). In this context, intrahost evolution of HIV-1 populations may be compatible with a Darwinian model system, as recently suggested (Bagnarelli et al., 1999; Ganeshan et al., 1997; Wolinsky et al., 1996).

The complete elucidation of the mechanisms driving intrahost HIV-1 evolu-
The V3 sequence is a variable domain in the HIV gp120 and contains 35 amino acids arranged in a loop. This domain plays a crucial role in driving important biological properties of the virus, including cell tropism. Generally, mutations in the V3 loop do not affect the ability of gp120 to interact with the CD4 receptor, although several studies have unambiguously indicated that V3 sequences play an important role in two correlated biological features with pathogenic implications, that is, syncytium formation (Willey et al., 1994) and...
coreceptor usage (Isaka et al., 1999). Importantly, analysis of chimeric viruses has revealed that changes in the V3 loop can convert a nonsyncytium inducing (NSI), slowly replicating virus into a syncytium inducing (SI), rapidly replicating virus (Shioda et al., 1992).

**CONCLUSION**

Expanded analysis of the molecular biology of HIV has been the key to understanding the mechanisms by which this virus persists in the host and causes AIDS, and to developing effective antiretroviral strategies. Application of powerful molecular biology tools has allowed researchers to obtain fundamental results on many aspects of HIV biology in vitro (i.e., in cell-free and tissue culture systems) and in vivo (i.e., directly in samples from the susceptible host). Importantly, knowledge of the molecular mechanisms in each step of the virus life cycle has provided an essential basis for discovering new antiviral compounds. Otherwise, a firm understanding of the relevant features of both the HIV turnover in vivo and the intrahost HIV evolution is crucial for developing effective anti-HIV strategies. Indeed, the HIV biology poses several challenges to the development of these strategies. In particular, sequence variation resulting from errors of the viral RT and recombination renders HIV an elusive target for both antiviral compounds and vaccines. In this context, novel diagnostic molecular tools to control development of viral resistance to the different classes of antivirals and new effective therapeutic approaches, including genetic and immunologic strategies, could be the key to inhibiting HIV replication in the future.

**REFERENCES**


References


Telomere Length, CD28− T Cells, and HIV Disease Pathogenesis

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INTRODUCTION

In human immunodeficiency virus (HIV) disease, as in all viral infections, CD8 T cells constitute a critical component of the protective immune response (Borrow et al., 1994; Brodie et al., 1999; Koup et al., 1994). Loss of CD8 T-cell activity coincides with the progression to acquired immunodeficiency syndrome (AIDS), and studies on long-term nonprogressors have underscored the importance of cytotoxic T lymphocyte (CTL) function (Cao et al., 1995; Goulder et al., 1997; Harrer et al., 1996). One of the intriguing alterations in the peripheral T-cell pool of individuals infected with HIV is the progressive accumulation within the CD8 T-cell subset of a population of cells that lack expression of the CD28 costimulatory molecule. Indeed, in some HIV-infected persons, >65% of the CD8 T cells are CD28−. A more complete characterization of this unusual cell population, therefore, is essential for understanding disease pathogenesis as well as for the development of appropriate strategies for treatment. Because CD28− T cells are poorly proliferative, do not contribute to production of soluble antiviral suppressive factors, and also show alterations in apoptosis and in cell-cell adhesion, the presence of large proportions of such cells will undoubtedly have a profound influence on the immune control over HIV infec-

This chapter is dedicated to my friend and colleague Janis V. Giorgi.
tion. Although there had been much speculation by AIDS researchers on the origin of the CD28\textsuperscript{−} T cells, elucidation of the nature of this expanded population of cells in HIV disease has emerged from research in a totally different arena of scientific investigation, namely basic cell biology studies on the process of replicative senescence. This chapter will review the findings that have led to the unexpected convergence of these two seemingly unrelated fields.

**REPLICATIVE SENESCENCE**

Normal human somatic cells have an intrinsic natural barrier to unlimited cell division. Following a fairly predictable number of cell divisions in culture, most, if not all, mitotically competent human cells reach an irreversible state of growth arrest known as replicative senescence, a process first identified by Hayflick in human fetal fibroblasts (Hayflick, 1965). Replicative senescence is a strict characteristic of human cells, and has, in fact, been proposed to constitute a tumor suppressive mechanism (Smith and Pereira-Smith, 1996). Interestingly, experimental cell fusion studies have demonstrated that the property of senescence is genetically dominant over immortality in a variety of human cell types, and spontaneous transformation of human cells in vitro rarely, if ever, occurs (Smith and Pereira-Smith, 1996). By contrast, most rodent cells have a high propensity to bypass senescence and transform spontaneously in culture (Campisi et al., 1996). The divergent behavior of human and mouse cells with respect to spontaneous immortalization in vitro suggests that conclusions regarding replicative properties, telomeres, and telomerase drawn from murine studies may not be applicable to human cells.

The characteristics of replicative senescence, or the so-called Hayflick Limit, have been explored in a variety of human cell types for more than 30 years, but only relatively recently has this model been applied to the immune system. Ironically, the Hayflick Limit may be particularly deleterious for immune cells, inasmuch as the ability to undergo rapid clonal expansion is absolutely essential to their function.

During the past decade, human T cells have been extensively characterized in cell culture models with respect to replicative senescence. A number of large-scale studies have shown that following multiple rounds of antigen, mitogen, or activatory antibody-driven proliferation, T cells reach a state of growth arrest that cannot be reversed by further exposure to antigen, growth factors, or any other established T-cell stimuli (Effros and Pawelec, 1997). The occurrence of replicative senescence has been documented for both clonal and bulk cultures of CD4 and CD8 T cells (Adibzadeh et al., 1995; Grubeck-Loebenstein et al., 1994; McCarron et al., 1987). It has also been shown that the replicative potential of memory CD4 T cells is reduced compared with naïve CD4 T cells from the same individual, a finding that is consistent with the notion that memory cells are the progeny of antigen-stimulated naïve T cells (Weng et al., 1995). It is important to emphasize that although cell cycle arrest is the most