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CELLULAR ASPECTS OF HIV INFECTION

CYTOMETRIC CELLULAR ANALYSIS

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Preface

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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1

HIV and Molecular Biology of the Virus-Host Interplay

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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 intergration 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 1.1. Genes of HIV, Proteins, and Function

HIV Gene	Protein	Function	Essential for Replication
gag	$Pr55^{gag}$	Polyprotein precursor for matrix protein (p17), capsid protein (p24), nucleocapsid protein p9, and p7	Yes
pol	$Pr160^{gag\text{-}pol}$	Polyprotein precursor for virion enzymes reverse transcriptase (RT)/RNAse-H (p51), protease (PR) (p10), and integrase (IN) (p32)	Yes
env	gp160	Polyprotein precursor for envelope glycoproteins gp120 and gp41 (receptor binding and membrane fusion, respectively)	Yes
tat	p14	Transcriptional transactivator (initiation and elongation of viral transcripts)	Yes
rev	p19	Regulates viral gene expression at post-transcriptional levels (regulates splicing and transport of viral RNAs from the nucleus to the cytoplasm)	Yes
nef	p27	Downregulates CD4 receptor, enhances virion infectivity, influences T-cell activation	No
vif	p23	Viral infectivity factor (infectivity reduced in *vif*-minus mutants)	No
vpr	p15	Virion protein (associated with the nucleocapsid) implicated in regulation of viral and cellular gene expression	No
vpu	p16	Influences virus release	No

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 3×10^{-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-

TABLE 1.2. HIV Cell Receptors and Their Natural Ligands

	CD4	CCR5	CXCR4	CCR3	CCR2b	BOB	BONZO
HIV							
NSI	+	+	−	+	+	+	+
SI	+	+	+	+	+	(+)	+
Natural ligands							
	MHC Cl. II	MIP-1α MIP-1β RANTES MCP-4	SDF-1	RANTES MCP-3 EOTAXIN	MCP-1 MCP-2 MCP-3	?	?

NSI, nonsyncytium inducing; SI, syncytium inducing.

tion is of crucial importance for understanding the natural history of this infection and developing effective anti-HIV strategies. In particular, the envelope glycoproteins of HIV-1 interact with receptors of the target cells and mediate the process of virus entry. This process is complex, including binding of the viral gp120 to CD4, conformational changes of the viral glycoprotein, and subsequent use of a coreceptor before gp41-mediated fusion of the viral envelope and the cellular membrane (Kwong et al., 2000; Rizzuto and Sodroski, 2000; Rizzuto et al., 1998; Wu et al., 1996; Wyatt et al., 1995; Zhang et al., 1999) (Table 1.2). The evolutionary changes characterizing the HIV-1 population during the natural history of infection strongly influence crucial regions of the viral *env* gene (Bagnarelli et al., 1999; Menzo et al., 1998; Salvatori et al., 1997; Scarlatti et al., 1997; Shankarappa et al., 1998, 1999; Wolinsky et al., 1996). Because different variable domains of the HIV-1 gp120 play a key role in driving the early steps of the viral infection cycle, including coreceptor usage (Isaka et al., 1999; Sato et al., 1999; Verrier et al., 1999; Xiao et al., 1998) and CD4 independence (LaBranche et al., 1999), careful analysis of the intrahost evolution of the HIV-1 *env* gene is strategic for addressing the relevant features of the virus-host relationships (Yamaguchi and Gojobori, 1997; Yamaguchi-Kabata and Gojobori, 2000). In addition, HIV entry is at present an attractive target for new classes of antiretroviral compounds (Sodroski, 1999); at present, these compounds include inhibitors of HIV binding to CCR5 and CXCR4 coreceptors and fusion inhibitors (Murakami et al., 1999; Ono et al., 1997; Sakaida et al., 1998; Torre et al., 2000).

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.

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2

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$^-$ 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

easily identifiable characteristic of replicative senescence, it is just one of a constellation of features associated with this cell state. T cells that have reached replicative senescence still retain some normal functions despite their inability to proliferate. For example, senescent CD8 T cells are capable of normal antigen-specific cytotoxic function and upregulation of CD25 (the alpha chain of the interleukin [IL]-2R) surface expression (Perillo et al., 1993). Similarly, senescent CD4 T cells secrete certain cytokines after stimulation via their antigen receptors in an antigen-specific fashion. Evidence has also been forthcoming for the acquisition of certain other properties and functions, including major histocompatibility complex (MHC, unrestricted cytotoxicity or natural killing), negative regulatory effects on other T cells (suppression), and expression of the CD4 receptor on CD8 T cells, making them double positive (Laux et al., 2000; Pawelec et al., 1986). Thus, senescence does not constitute a general breakdown of normal function, but rather may comprise selected genetic and phenotypic alterations, resulting not only in loss, but also in gain, of function. It has been proposed, therefore, that replicative senescence may, in fact, represent a developmentally programmed state of terminal differentiation in T cells (Globerson and Effros, 2000).

Several important genetic changes are associated with T-cell replicative senescence in cell culture. For example, CD8 T-cell senescence is associated with increased expression of bcl 2 and resistance to apoptosis (Spaulding et al., 1999), changes that have also been observed in cultures of senescent fibroblasts (Wang et al., 1994). Another genetic feature associated with replicative senescence in T cells is the dramatically reduced transcription of gene for the major heat shock protein, HSP70, in response to stress (Effros et al., 1994b). Arguably, the most dramatic change associated with T-cell replicative senescence in cell culture is the complete loss of cell surface (and mRNA) expression of the CD28 costimulatory molecule (Effros et al., 1994a; Pawelec et al., 1997). The complete absence of CD28 on the cell surface at senescence is in marked contrast to the quantitative modulation in the level of CD28 expression that accompanies the process of activation. The loss in CD28 expression in cultures that reach replicative senescence is also remarkable in light of the unchanged expression of a variety of other T-cell markers reflecting lineage, activation, memory status, and adhesion (Perillo et al., 1993).

CD28 is a 44-kD disulfide-linked homodimer expressed constitutively on the majority of mature T cells whose signaling is essential for full T-cell activation. Ligation of the T-cell antigen receptor without costimulation by CD28 ligands such as the "B7" proteins on the surface of antigen-presenting cells (APCs) results in anergy, an unresponsive state in which the cells are unable to enter cell cycle. CD28 signal transduction results in IL-2 gene transcription, expression of the IL-2 receptor, and the stabilization of a variety of cytokine messenger RNAs (June et al., 1994). CD28 has additional biologic functions, which include mediation of protective effects against septic shock in vivo, influencing the class of antibodies produced by B cells, and enhancing T-cell migration and homing (Shimizu et al., 1992).

One of the newly identified roles of CD28 is its involvement in the induction of the enzyme telomerase. Telomerase is a specialized reverse transcriptase that functions to extend telomere sequences at the ends of linear chromosomes. In the absence of telomerase, telomeres, the repetitive DNA sequences on the ends of chromosomes, shorten with each cell division (Harley et al., 1990). In tumor cells, germ cells, and primordial stem cells, constitutive telomerase activity presumably prevents telomere shortening and allows unlimited cell division (Counter et al., 1994; Kim et al., 1994). This observation led to the theory that telomerase activity was absent in all normal somatic cells and present only in tumor cells. Indeed, extensive analyses of in vivo–derived tumor cell samples confirmed the presence of telomerase in >90% of tumors and its absence in the adjacent normal cells from the same patient (Shay and Wright, 1996). Telomerase activity, therefore, is believed to enable tumor cells to divide indefinitely. Conversely, because telomerase is absent from most normal somatic cells, it has been proposed that telomere shortening is causally linked to replicative senescence (Harley et al., 1990). However, it has recently become clear that the relationship of telomeres, telomerase, and senescence is far more complex in T lymphocytes than in other cell types.

Cells of the immune system differ dramatically from fibroblasts and most other normal human somatic cells in that, under certain circumstances, they exhibit telomerase activity levels similar to those observed in tumor cells. For example, robust telomerase activity has been documented in tonsillar B cells, developing T cells within the thymus, and in lymphoid organs (Weng et al., 1996, 1997b). In addition, high levels of telomerase activity that correlate with telomere length maintenance are observed in recently activated T cells (Bodnar et al., 1996; Weng et al., 1997b). The activation-induced telomerase in T cells is further enhanced by CD28 signaling (Weng et al., 1996), and conversely, telomerase up-regulation in antigen-specific human T cells can be blocked by treatment of the APCs with antibodies that block the CD28/B7 interaction (Valenzuela and Effros, 2000b). Interestingly, antigen-specific T cells that are repeatedly reactivated by exposure to antigen over time in long-term culture eventually lose the ability to up-regulate telomerase and senesce in culture (Valenzuela and Effros, 2000b). If a similar down-regulation of telomerase inducibility occurs in vivo, this could explain the presence of CD8 T cells with shortened telomeres in situations of chronic antigenic exposure.

Telomere length measurement provides an extremely powerful tool for analysis of both the replicative history and the future replicative potential of most cells. In the absence of telomerase, telomere shortening occurs with each cell division (Allsopp et al., 1992; Harley et al., 1990). Telomere analysis on cell cultures of both fibroblasts and T cells has demonstrated that telomeres shorten by approximately 100 bp per population doubling until reaching senescence (Vaziri et al., 1993). The specific link between telomere length and cell cycle arrest, the so-called telomere hypothesis of replicative senescence, was supported by several landmark studies published in early 1998. Specifically, it was shown that in fibroblasts and pigmented retinal epithelial cells, the enforced

constitutive expression of the catalytic component of human telomerase (hTERT) gene enabled cells to maintain telomere length and continue dividing indefinitely (Bodnar et al., 1998; Vaziri and Benchimol, 1998). The seemingly immortal cells appear to be normal in every way, allaying fears that bypassing replicative senescence might automatically lead to malignant transformation (Jiang et al., 1999). Whether similar manipulations in T cells will lead to parallel outcomes is currently being investigated in several laboratories.

TELOMERE LENGTHS AND HIV DISEASE

Identification of the specific telomere sequence led to the rapid development of a variety of techniques to evaluate telomere length. Telomeres can be measured by isolating total genomic DNA from a population of cells, incubating this DNA with restriction enzymes that digest all but the telomeric and subtelomeric sequences, and perfoming Southern analysis (either gel or slot blot) with radiolabeled telomeric probe (Bryant et al., 1997; Valenzuela and Effros, 2000a). The results of such analyses provide the mean terminal restriction fragment (TRF) length of a cell population, which, in turn, reflects the composite of the telomere lengths of all the chromosomes in each of the cells in the starting population. Cell sorting techniques to enrich for particular cell subpopulations (Effros et al., 1996; Monteiro et al., 1996), analysis of TRF lengths from sorted chromosomes (Martens et al., 1998), or probing specific chromosomes containing telomeres that have been seeded with plasmids (Murnane et al., 1994) may, in some cases, increase the precision of the telomere assay. A second strategy, developed by Landsorp and colleagues, uses fluorescence in situ hybridization (FISH) technology on metaphase spreads or on interphase nuclei, with flow cytometric visualization and quantitation of the amount of telomere sequences on individual chromosomes (Lansdorp et al., 1996). Flow cytometric analysis of telomere size in concert with cell cycle analysis adds an additional dimension to telomere studies (Hultdin et al., 1998). Finally, techniques currently under development that combine FISH telomere analysis with flow cytometric cell surface phenotype or tetramer-binding will provide critical information on specific subpopulations of cells. Telomere length assays have contributed remarkable insights into a variety of facets of cell biology in both normal and transformed cells. The specific telomere sequence also forms the basis for assays to detect telomerase, using techniques involving telomere repeat amplification protocol (TRAP) (Kim et al., 1994).

In the context of HIV disease, telomere length analysis has been used as a tool to analyze the dynamics of lymphocyte homeostasis and its relation to ultimate immune collapse that is fundamental to HIV disease pathogenesis. Research in this area has provided extraordinary insights into both T and B function in HIV-infected persons, as will be described below.

In a longitudinal analysis of peripheral blood mononuclear cell (PBMC) samples collected over a period of 9 years, Miedema and colleagues docu-

mented that telomere lengths of total PBMC from HIV-infected individuals shortened at an accelerated rate compared with age-matched seronegative controls (Wolthers et al., 1996). For HIV-infected individuals, the mean TRF length loss in the progressors (175 ± 105 bp/yr) was greater than that in the asymptomatic individuals (114 ± 100 bp/yr), and both were significantly increased compared with healthy controls (4.7 ± 71 bp/yr). These data indicate that increased cell division of immune cells is associated with HIV infection and correlates with disease status.

Telomere length measurements on defined cell populations separated by cell sorting procedures have provided further insight into HIV disease pathogenesis. Several groups compared telomere lengths of the two T-cell subsets. In four independent studies, telomere shortening was consistently observed within the CD8 T-cell subset, with only minimal or no telomere shortening in the CD4 subset. In one study, sequential samples from several individuals demonstrated progressive CD8 T-cell telomere shortening over time (Wolthers et al., 1996). In another study, the CD8 T cells were further sorted, permitting the demonstration that within the CD8 subset, all of the telomere shortening could be accounted for by the cells that lacked CD28 expression (Effros et al., 1996). In fact, the telomere lengths of the CD8$^+$CD28$^-$ T cells were the same size as those of centenarian lymphocytes, suggestive of accelerated immunological aging during HIV disease. Boussin and colleagues (Pommier et al., 1997) have also documented CD8 telomere shortening and, furthermore, provide data showing that B-cell telomeres shorten as well, consistent with the well-documented hyperactivation and polyclonal antibody production observed during HIV infection. Finally, a novel approach to telomere analysis in the two T-cell subsets was used by Hodes and colleagues (Palmer et al., 1997) in an effort to eliminate the variability inherent in telomere lengths in outbred human populations by comparing cell populations derived from HIV-discordant identical twins. These studies showed that the mean TRF of the CD8 T cells was shorter in HIV-infected versus uninfected twins (mean difference 1.1 ± 0.7 kb). Thus, accelerated telomere shortening in CD8 T cells during HIV disease has been an unequivocal observation in all of the published studies that have used this experimental approach.

Numerous studies have been performed in an effort to elucidate CD4 T-cell dynamics in HIV disease, but, in marked contrast to the CD8 T-cell findings, the results have been inconsistent, showing an entire spectrum of changes, possibly suggesting a more complicated picture. For example, in the same twin study in which CD8 T-cell telomeres were shorter in the infected twin, Palmer et al. (Palmer et al., 1997) demonstrated the CD4 T cells in the HIV-infected twins were longer than those in the uninfected twin (mean difference 0.9 ± 0.4 kb). Furthermore, the CD4 T-cell subset showed no difference in the total number of population doublings achieved in long-term culture between the HIV-infected versus uninfected twins. The data from these studies were interpreted to indicate that the immune deficiency associated with HIV disease could not be attributed to exhaustion of the replicative potential of CD4 T cells. Even in studies that do document some CD4 T-cell telomere shortening, these

changes do not correlate with markers of disease progression. Preliminary evidence from two studies had suggested that CD4 telomere shortening might occur at late stage disease (Effros et al., 1996; Pommier et al., 1997). However, in a subsequent more extensive analysis of TRF in PBMC versus CD4 T cells, both increases and decreases of CD4 T-cell telomeres were observed, with no correlation between CD4 T-cell telomere length changes and other well-established markers of disease status, such as CD4 counts and $CD8^+CD38^+$ T cells (Effros, Giorgi, Valenzuela, and Mitsuyasu, manuscript in preparation).

DIVERGENT TELOMERE DYNAMICS BETWEEN CD8 AND CD4 T CELLS IN HIV-INFECTED PERSONS

Telomere measurement, at best, can only provide information on the replicative history and the long-term total replicative potential of a cell population, but is not the most appropriate assay for studying overall population dynamics, half-life, or rate of replacement of T cells in HIV disease. Thus, the marked divergence in the telomere findings between CD8 and CD4 T cells need not be interpreted as being at odds with the cell turnover studies, but rather as further demonstration of the restricted information that can be derived from telomere length measurement. Indeed, cell turnover studies that measure the rate of cell division have clearly demonstrated quite similar rates for both T-cell subsets. For example, equivalently high turnover in both CD4 and CD8 T cells from HIV-infected persons has been determined by analysis of the mutation frequency at the hypoxanthine phosphoribosyltransferase (HPRT) locus (Paganin et al., 1997) and in bromodeoxyuridine labeling studies on simian immunodeficiency virus-infected rhesus macaques (Mohri et al., 1998; Rosenzweig et al., 1998). Thus, the extraordinarily high turnover rate of CD4 T cells, a notion based on the rebound of CD4 T-cell number during antiretroviral therapy (Ho et al., 1995; Wei et al., 1995), need not necessarily involve telomere shortening and, therefore, the telomere length findings do not conflict with studies measuring repopulation, replacement, or rate of cell division. Moreover, in the absence of telomere data on specific CD4 T-cell subpopulations defined by phenotypic markers, precise conclusions from telomere studies are impossible. Finally, even research involving clonal populations of T cells propagated in vitro indicates that the unconditional use of telomere length data to assess turnover of lymphocytes may be lead to incorrect conclusions (Rufer et al., 1998).

The divergence in observed telomere shortening patterns between CD4 and CD8 T cells, therefore, most probably reflects the disparate roles of the T-cell subsets during HIV disease rather than any inherent limitations of the technique of telomere analysis itself (Hellerstein and McCune, 1997; Rosenzweig et al., 1998). Because CD4 T cells are targets of HIV, it seems likely that those cells that become infected by HIV undergo only a few cell divisions before dying by apoptosis or being lysed by CTL, thereby limiting the time frame during which telomere shortening can occur. Alternatively, when activated

CD4 T cells are either killed or undergo apoptosis and are replaced by thymus-derived naïve cells, the longer telomere lengths of the naïve cells may obscure any CD4 T-cell shortening that might have occurred among memory CD4 T cells. Efforts aimed at optimizing Flow-FISH telomere analysis will ultimately clarify these issues.

The potential involvement of telomerase in the different telomere length dynamics of CD4 and CD8 T cells provides another possible explanation for the observations in HIV-infected individuals. Telomerase, the enzyme that extends telomere sequences, might function to retard or prevent telomere shortening in CD4 T cells. Although no differences in baseline or inducible telomerase levels were detected in vitro between CD4 and CD8 T cells during the chronic stage of infection (Effros et al., 1996; Wolthers et al., 1996, 1999), these findings do not preclude possible in vivo involvement of telomerase activity at an earlier time-point in the disease process. Telomere length maintenance is, in fact, observed in human T cells in cell culture during the period that coincides with high telomerase activity (Bodnar et al., 1996). Similarly, a study on the kinetics of telomerase induction in vivo during the very early stage of Epstein-Barr virus (EBV) infection shows that high telomerase activity is present in antigen-specific T cells during acute infectious mononucleosis and correlates with elongation of telomeres in these cells (Maini et al., 1999). This study suggests that telomere-lengthening mechanisms can operate in vivo during the early stages of infection and that there is apparent lengthening within the population of T cells that is undergoing massive clonal expansion. Germinal center B cells with elongated telomeres have also been reported (Weng et al., 1997a). Thus, results from other experimental systems are consistent with the possibility that CD4 T cells in HIV-infected persons have indeed undergone a greater number of cell divisions than is reflected in their telomere length.

In any event, whatever factors contribute to the absence of the expected telomere shortening in the CD4 subset, one should not dismiss the importance of the dramatic and progressive telomere shortening in CD8 T cells, the subset responsible for effective immune control over the virus. More importantly, because telomere length is, in general, predictive of a cell's overall proliferative potential (Harley et al., 1990), the telomere studies on HIV-infected persons document the presence of a population of CD8 T cells that is severely compromised in its ability to undergo further clonal expansion. In addition, the high proportion of CD28$^-$ T cells will clearly reduce a critical component antiviral effector function, because antiviral suppressive factor production is restricted to CD8 T cells that are CD28$^+$ (Landay et al., 1993).

TELOMERE STUDIES AND HIV PATHOGENESIS

As discussed above, the absence of telomere shortening in CD4 T cells has numerous potential explanations. Unfortunately, the substantial effort expended on the analysis and debate regarding *differences* in telomere dynamics between

the two T-cell subsets has diverted attention of AIDS biologists from the fundamental significance of shortened telomeres in the CD8 T cells. Indeed, it is this aspect of the AIDS telomere studies that might provide the greatest insight into disease pathogenesis. In fact, although unbeknown to the community of AIDS researchers, cell biologists interested in the mechanisms controlling cell cycle arrest, telomere regulation, and the Hayflick Limit view HIV disease as the most dramatic example to date of the in vivo pathophysiologic relevance of the process of replicative senescence.

Our own telomere studies were, in fact, originally initiated to specifically test whether replicative senescence might be occurring during HIV disease. We had already documented T-cell telomere shortening during aging, had identified loss of CD28 expression as a marker of replicative senescence in vitro, and had reported age-related increase in the proportion of CD8 T cells lacking CD28 expression (Boucher et al., 1998; Effros et al., 1994a; Vaziri et al., 1993). Interestingly, it has been recently suggested that loss of CD28 expression in vivo might be a marker of chronic/latent infection, based on the correlation of increased proportions of $CD28^-$ T cells with chronic CMV infection (Looney et al., 1999; Weekes et al., 1999b). High proportions of $CD28^-$ T cells had been documented in HIV-infected individuals, but their origin and significance were unclear (Borthwick et al., 1994; Brinchmann et al., 1994; Jennings et al., 1994; Lewis et al., 1994). When we compared telomere lengths in sorted populations of $CD28^+CD8^+$ and $CD28^-CD8^+$ T cells obtained from individual HIV-infected donors, we found that the telomere length of the $CD28^-CD8^+$ T-cell subset had shortened to a size of 5–7 kb, which is identical to that of T cells that have undergone multiple rounds of antigen-driven proliferation in cell culture to reach replicative senescence (Effros et al., 1996). Moreover, within individual donors, the proportion of $CD28^-$ T cells increases progressively over time. Because T cells lacking CD28 are nonproliferative in response to anti-CD3 antibodies plus IL-2 (Azuma et al., 1993) or phorbol myristic acetate/ionomicin plus IL-2 (Effros et al., 1996), our findings supported the hypothesis that replicative senescence might be a feature of HIV disease pathogenesis.

The demonstration of extensive CD8 T-cell division possibly leading to the irreversible state of replicative senescence in HIV disease builds on an increasing body of evidence suggesting a crucial role for CD8 T cells in both the primary acute response as well during disease progression. Giorgi and others have demonstrated that cellular markers of immune activation, especially increased expression of CD38 and HLA-DR on the surface of CD8 T cells, accompany, reflect, and predict disease progression. (Cossarizza et al., 1995; Giorgi et al., 1994, 1999; Ho et al., 1993; Kaufmann et al., 1999; Zaunders et al., 1999). Diminished expression of CD38, correlating with increased proportions of $CD28^+$ T cells and increased proliferative capacity, has been documented in patients receiving long-term highly active antiretroviral therapy (HAART) (Carcelain et al., 1999; Sndergaard et al., 1999), suggesting that CD28 expression may be an important marker of immune function. In this regard, it is becoming increasingly clear that the combination of immunological and virologic

markers may provide increased precision in monitoring both disease progress and response to antiretroviral therapy (Roos et al., 1998). The notion of replicative senescence of HIV-specific CD8 T cells is also consistent with and expands upon the seminal finding of Carmichael et al. (1993) showing that HIV-specific memory precursors are lost as HIV disease progresses despite retention of memory cells specific for other pathogens. The limiting dilution assays used in that study are critically dependent on the ability of cells to undergo clonal expansion in vitro. The absence of detectable HIV-specific memory precursors when considered together with our telomere data suggest that the expanded population of CD28$^-$CD8$^+$ T cells might reflect a specific immune response to HIV (Giorgi et al., 1995; Landay et al., 1983).

The telomere shortening and the presence of CD28$^-$ T cells observed in HIV disease is a more dramatic example of events that also occur in healthy individuals, and may possibly reflect a common pattern of T-cell dynamics with respect to pathogens in general. In HIV-seonegative individuals, the proportion of T cells lacking CD28 expression increases progressively with age, from 1% in newborns to levels of 35% in centenarians (Azuma et al., 1993; Boucher et al., 1998). Interestingly, at all ages, cells with this phenotype are significantly more prevalent within the CD8 versus CD4 subset. The notion that at least some of these expanded clones were originally generated during acute antiviral responses is supported by observations in mice that secondary responses are composed of the same clonotypes dominating the primary responses (Maryanski et al., 1996). In humans, maintenance of specific T-cell receptor (TCR) clonotypes at high frequencies in the circulating CD8 T cells has been observed in numerous infections, including chronic HIV infection, cytomegalovirus, and influenza (Callan et al., 1998; Kalams et al., 1994; Wills et al., 1996). In acute infectious mononucleosis, $\sim$40% of the CD8 T cells are specific for a single viral epitope (Callan et al., 1998), and many of the expanded clones seen in the primary response can be detected one year later directly ex vivo using the heteroduplex technique. Further evidence of long-term maintenance of virus-specific T cells emerges from studies on EBV, where the CTL generated in vitro upon restimulation with autologous EBV-transformed B cells contain clones that had been detected in vivo at the onset of infection.

REPLICATIVE SENESCENCE AND HIV DISEASE

It is impossible to formally prove that replicative senescence is occurring in vivo during HIV disease. In a closed cell culture system, where the same population of antigen-specific T cells can be followed over its entire replicative lifespan, one can be fairly certain that CD28$^-$ T cells with telomeres of 5–7 kb are the progeny of antigen-specific CD28$^+$ T cells with telomeres of 11 kb that were used to initiate the culture (Effros, 1998). Indeed, the generation of CD28$^-$ T cells from long-term stimulated CD28$^-$CD8$^+$ T cells has been documented in cell cultures initiated from T cells isolated from both HIV-infected and unin-

fected donors (Fiorentini et al., 1999; Posnett et al., 1999). However, in human subjects, one can never conclusively prove that the $CD28^-$ T cells arose by the same mechanism. Nevertheless, the presence of identical HIV-specific clonotypes in the $CD28^+$ and $CD28^-$ T cells has led several investigators to suggest a common lineage for the two cell populations (Dalod et al., 1999; Mugnaini et al., 1998).

Functional studies provide additional evidence that is consistent with the occurrence of replicative senescence during HIV disease progression. The proliferative defects and apoptosis resistance, first documented in cell culture, are characteristic of ex vivo–derived $CD28^-CD8^+$ T cells from HIV-infected persons (Posnett et al., 1999). Furthermore, when PBMC derived from HIV-infected individuals are compared to PBMC that have been depleted of the $CD28^+$ T cells, the precursor CTL (pCTL) frequency for several HIV peptides is the identical in the two cell populations (Weekes et al., 1999b). Because the removal of the T cells that express CD28 caused no reduction in the frequency of pCTL, these studies demonstrate that the $CD28^-$ T cells must be the major source of the antigen-specific CTL function. As mentioned above, robust antigen-specific cytolytic function is a hallmark of CD8 T cells that reach replicative senescence in cell culture. Indeed, the antigen-specific cytotoxic function of senescent cultures is even more potent than that of the $CD28^+$ T cells from which they were derived (Perillo et al., 1993). Thus, $CD28^-$ T cells, whether generated in long-term cultures or tested ex vivo from HIV-infected individuals, show specific proliferative defects, cannot be induced to express telomerase activity, have telomeres of 5–7 kb, show potent antigen-specific cytotoxicity, and are resistant to apoptosis, findings that lend support to the idea that the process of replicative senescence may be occurring within the CD8 T-cell subset during chronic HIV infection.

Elucidation of the nature and etiology of the $CD28^-CD8^+$ T cells that accumulate in HIV disease is not merely an academic exercise. Insight into the origin of these cells will provide important information on both disease pathogenesis as well as appropriate strategies for treatment. It is well-documented that the $CD8^+$ T cells in HIV disease are a critical component of the protective immune response (Borrow et al., 1994; Brodie et al., 1999; Koup et al., 1994). Studies on long-term nonprogressors have highlighted the importance of CTL function, and the loss of CD8 T-cell activity coincides with the progression to AIDS (Cao et al., 1995; Goulder et al., 1997; Harrer et al., 1996). In addition to this decrement and the limitation posed by $CD28^-$ T cells on the process of clonal expansion, because the CD28 molecule also functions to increase the avidity of cell adhesion (Shimizu et al., 1992), cells lacking CD28 may have altered trafficking and homing patterns. Altered expression of other adhesion molecules is, in fact, a feature of HIV disease (Park et al., 1998; Schmitz et al., 1998). Another functional characteristic of $CD28^-CD8^+$ T cells that might impact disease progression is the altered generation of hydrogen peroxide as well as catalase levels (Yano et al., 1998). Thus, the increasing proportion of CD8 T cells that is hampered in its ability to undergo further clonal expansion,

produce soluble antiviral factors, generate sufficient antioxidants, and traffic normally into the tissues will undoubtedly diminish the quality of the protective immune response. Admittedly, it is possible to argue that the above characteriistics, rather than being abnormal, reflect the evolution of a mature immune response. Namely that the CD28$^+$CTL precursors first proliferate and secrete antiviral factors, and, as they divide, an increasing proportion of cells lose CD28 expression, have shortened telomeres, and the amount of antiviral factors decreases as the cytotoxicity is enhanced and reaches its peak at the point at which the CD8 T cells can no longer undergo proliferation. This alternative scenario, however, seems less likely, inasmuch as the increase in the proportion of CD28$^-$ T cells correlates with disease progression and ultimate *loss* of control over the infection. More detailed elucidation of what seem to be discrete stages CD8 T-cell effector cell progression in HIV-infected persons seem to be emerging from approaches using additional markers such as CD27 and CD57 to more precisely define CD8 T-cell function and cytokine profiles (Appay et al., 2000; Lewis et al., 1999). Such analyses will provide more detailed understanding of the biology of CD8 T cells during chronic infection and their precise contribution to HIV disease pathogenesis.

Irrespective of the specific mechanism responsible for the progressive increase in the proportion of CD28$^-$CD8$^+$ T cells in HIV-infected persons, the physical presence of this substantial population will undoubtedly influence homeostatic processes that control the size and components of the peripheral T-cell pool (Caruso et al., 1998; Freitas et al., 1996; Rocha et al., 1989). What is already clear from studies on elderly people not infected with HIV is that an increased proportion of CD8 T cells lacking CD28 is associated with reduced numbers of naïve CD8 T cells (Fagnoni et al., 2000), indicating a possible negative feedback regulatory mechanism that is subset specific. Moreover, in HIV-infected persons, several groups have documented an inverse correlation between numbers of CD28$^-$CD8$^+$ and CD4 T cells (Caruso et al., 1998; Choremi-Papadopoulou et al., 2000; Lewis et al., 1999). In this regard, it is possible, for example, that the removal of some of the CD28$^-$CD8$^+$ T cells might serve as a stimulus for the production of more functional antigen-specific CD8 T cells, and possibly even new CD4 T cells. Although it is highly unlikely that current antiretroviral treatment regimens will reverse telomere shortening and loss of CD28 expression in individual cells, use of these drugs in combination with the physical removal of senescent cells may prevent a recurrence of the excessive telomere shortening, replicative senescence, and loss of CD28. Antiretroviral therpay is, in fact, associated with the reconstitution of the lymphoid compartment with cells having longer telomeres, indicative of increased replicative potential (Kaushal et al., 1999). Early implementation of antiretroviral therapy may also prevent the telomerase inhibition observed in bone marrow stem cells of HIV-infected persons (Vignoli et al., 1998), a process that may be further accelerating the disease progression and immune collapse.

The development of new treatment approaches for HIV disease can also be profoundly influenced by increased understanding of the nature of the

$CD28^{-}CD8^{+}$ T cells. If these cells are simply anergic, as has been suggested (Lewis et al., 1994), alternative and enhanced costimulatory strategies may be required to reverse or overcome the unresponsive state. However, if these cells represent the end stage of replicative senescence, as we propose, gene therapy techniques such as those that have reversed senescence in other cell types may be more promising approaches. Moreover, vaccine protocols that target CD8 T cells may also require consideration of possible strategies to ensure that T-cell protection is not diminished over time by exhaustion of proliferative potential of antigen-specific CD8 T cells.

CONCLUSION

Telomere studies have highlighted a previously unrecognized aspect of CD8 T-cell biology occurring during HIV disease. The demonstration that the non-proliferative $CD28^{-}CD8^{+}$ T-cell supbpopulation with telomeres in the identical 5–7 kb range, previously associated with replicative senescence reaches levels of >65% in HIV-infected persons, further underscores the biological relevance of these observations. In light of the increasing recognition of the importance of the protective role of CD8 T-cell in HIV disease, it seems clear that research aimed at retarding CD8 T-cell telomere shortening and delaying replicative senescence might lead to novel immunotherapeutic strategies that will complement treatments aimed at the virus itself.

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3

Immune Dysregulation and T-Cell Activation Antigens in HIV Infection

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INTRODUCTION

Human immunodeficiency virus (HIV) infection is associated with a complex pattern of alterations that profoundly affect the immune response (Cohen et al., 1997; Levy, 1983; Shearer, 1998). HIV-induced immune dysregulation impairs both the quantitative and qualitative homeostasis of the immune system, and this impairment is manifested as an array of multiple, characteristic defects that can be summarized as follows:

- A progressive decline in $CD4^+$ T-lymphocyte numbers and a profound impairment of the functionality of these cells
- A decrease in interleukin (IL)-2, IL-12, IL-15, and interferon (IFN)γ (type-1 cytokines that mainly stimulate cell-mediated immunity and immune defenses against intracellular pathogens) and an increase in IL-4, IL-5, IL-6, and IL-10 production (type-2 cytokines that mainly stimulate antibody production and humoral immunity and are finalized at the activation of immune defenses against extracellular pathogens)
- Changes in immunophenotype of HIV-infected cells (Table 3.1):
  - *$CD4^+$ T cells* increase in the subpopulations expressing CD45RO, Fas

TABLE 3.1. A Brief Summary of the Main Changes Observed When Immunophenotyping Is Performed in Cells of HIV-Infected Patients

	CD45RO	CD45RA	CD95	CD25	CD38	HLADR	CD57	CD69	CD7	SLAM
$CD4^+$	↑	↓	↑	↑	↑	↑	↑	↑	↓	↓
$CD8^+$	↑	↓	↑	↓	↑	—	↑	↑	—	—

(CD95), CD25, CD38, HLA-DR, CD57, and CD69 and decrease in the subpopulations expressing CD45RA, CD7, and signaling lymphocytic activation molecule (SLAM)

- *$CD8^+$ T cells* increase in the subpopulations expressing CD38, CD45RO, CD57, and CD69 and decrease in the subpopulations expressing CD45RA and CD28
- *NK cells* increase in the subpopulations expressing CD56 and decrease in the CD16/CD56 subpopulations

HIV infection ultimately results in the appearance of the acquired immunodeficiency syndrome (AIDS); the diagnosis of AIDS was, until the introduction of highly active antiretroviral therapy (HAART), shortly thereafter followed by death.

In this chapter we will briefly summarize the defects in T-helper functions and phenotype induced by HIV infection as well as the effects of HAART on immune reconstitution.

FUNCTIONAL IMMUNE DYSREGULATION IN HIV INFECTION

Primary HIV infection can be asymptomatic or can be associated with a flu-like syndrome characterized by fever, malaise, and weakness (Kahn and Walker, 1998). The early phase of HIV infection is also always correlated with the presence of extremely elevated titers of HIV in the plasma (Ho et al., 1994). A potent HIV-specific immune response is rapidly induced within a few days after primary infection. In this phase, both humoral immunity (HI) and cell-mediated immunity (CMI) are promptly stimulated. It was nevertheless convincingly demonstrated that a significant reduction in HIV plasma viremia depends on the generation of an HIV-specific cell-mediated immune response, independently of the magnitude of the humoral immune response (Borrow et al., 1994; Poignard et al., 1999). This concept is mostly based on the observation that the detection of HIV-specific cytotoxic T lymphocyte (CTL) precedes reductions in viral load, whereas the generation of neutralizing antibodies is delayed and is observed after the changes in HIV viremia has taken place (Borrow et al., 1994). The idea that the modulation of HIV replication is associated with an intact and powerful HIV-specific CMI (Clerici and Shearer,

1993, 1994) at least in part derives from the observation that HIV replication is relatively controlled in the initial phases of the infection, when the immune system is powerful and still relatively undamaged.

A long period of clinical asymptomaticity follows primary HIV infection. This phase is initially characterized by low HIV viral load, reduced HIV replication in peripheral blood mononuclear cells (PBMC), and low HIV isolability from PBMC (Levy, 1993). In this period, HIV replicates vigorously within the lymphatic tissues (Pantaleo et al., 1993). CD4 T-helper cell functions are nevertheless often disrupted even in the earlier phases of asymptomatic HIV infection (Clerici et al., 1989). The defects detected in T-helper function of asymptomatic, HIV-infected individuals are epitomized by the impairment of the ability of these cells to proliferate and secrete IL-2 in response to nominal antigens (Clerici et al., 1989). The T-helper defects observed in this phase of the disease are independent of CD4 counts (Clerici et al., 1989) and predictive for the subsequent rate of decline in the number of $CD4^+$ T lymphocytes (Dolan et al. 1995), time to onset of AIDS, and time to death (Dolan et al. 1995). Abnormalities in T-helper integrity include, besides the defects in IL-2 production, a series of other alterations, among which reduced IL-2 receptor expression (Prince et al., 1984), preferential loss of the naive T-cell subset (Roeder et al., 1995), and down-regulation of the expression of the ζ chain of the T-cell receptor (Trimble and Lieberman, 1998) may have an important role in disease progression.

In the period of clinical asymptomaticity, the ability of T lymphocytes to produce cytokines other than IL-2 upon antigenic stimulation is impaired as well, and this impairment appears to be pivotal in the progression of the infection. To summarize, in vitro stimulation of blood leukocytes from HIV-infected patients results in decreased production of IL-2, IFNγ, and IL-12 and in increased production of IL-4, IL-5, IL-6, and IL-10 (Clerici and Shearer, 1993, 1994). Decreased IL-2 production and increased IL-10 generation (and the IL-10/IL-2 ratio) are associated with the isolation of syncytium-inducing (X4) strains of HIV (Clerici et al., 1996a), whereas the opposite scenario (potent type-1 cytokine secretion and weak production of type-2 cytokines) is correlated with a condition of long-term nonprogression (Clerici et al., 1996b). Thus, even in the period in which clinical latency is observed, HIV infection is immunologically and virologically extremely active and destructive. From a virological point of view, the remarkably fast rate of HIV replication, and the enormous ability of this virus to modify in response to exogenous (pharmacologic) and endogenous (immune system) selective pressure, is underlined by the observation that a wild-type, drug-susceptible HIV strain can be totally replaced by a mutant, drug-resistant strain within 2–4 weeks after initiation of antiretroviral therapy (Ho et al., 1994).

The end of the period of clinical latency coincides with the onset of signs and symptoms of AIDS, the rapid destruction of $CD4^+$ T lymphocytes, and a significant increase in HIV viral load.

T-CELL ACTIVATION ANTIGENS DURING HIV INFECTION

Immune activation was early recognized as being a characteristic feature of HIV infection. Because HIV replicates in activated cells (Zack et al., 1988, 1990), the observation that an abnormal and augmented immune activation takes place in this disease has an obvious, immediate clinical relevance. Additionally, immune-activated cells will be unable to respond to antigens, might produce reduced amounts of soluble antiviral factors, and are more susceptible to programmed cell death. A vast array of immune cells can phenotypically be characterized as being activated in HIV-infected individuals, and activation is seen in virtually each immune cell compartment. Thus, $CD4^+$ and $CD8^+$ T lymphocytes as well as natural killer (NK) cells show signs of immune activation. Markers of T-cell activation in HIV infection include, among others, increased cell surface expression of CD45RO, CD25, CD28, CD38, HLA-DR, and CD95 (Giorgi and Janossy, 1994).

CD45RO and CD45RA

In adult individuals with a normal immune system who are not infected with HIV, the majority of T cells are memory cells that have been exposed to antigen, although up to 45% of CD4 cells may be naive (Autran et al., 1997). Naive T cells express the CD45RA as well as other markers including CD62L (L-selectin) and CD44 marker (Farber, 2000; Sprent, 1994). This results in different homing patterns, with naive cells preferentially migrating to lymph nodes and memory cells more prone to migrate into peripheral tissues, particularly when an inflammatory process is present (Farber, 2000; Sprent, 1994). Although published estimates vary, a current estimate for the turnover rate of naive T cells in non-HIV-infected individuals is several (3.5–6) years. Because the death rate of naive cells is suggested to be very low, this turnover is probably mainly associated with cell division and maturation (Finzi and Siliciano, 1998; Hellerstein and McCune, 1997). Resting, naive CD4 T cells are not very susceptible to HIV infection (Finzi and Siliciano, 1998; Hellerstein and McCune, 1997), whereas the activated naive T cell ($CD45RA^+$), which has a lifetime of several weeks after antigen stimulation, is extremely susceptible to HIV infection (Finzi and Siliciano, 1998; Hellerstein and McCune, 1997). After most of these cells have died, a few long-lived memory CD4 cells that are ready to be activated by specific peptide–major histocompatibility complexes persist in a resting state, with a turnover time (0.4–1.1 years) that is shorter than the characteristic of naive T cells (Cossarizza, 1997). Resting and activated memory ($CD45RO^+$) T cells are therefore the primary targets of HIV infection (Cossarizza, 1997), but, despite these cells being preferential or selective targets for HIV, the proportion of naive/memory CD4 cell ratio decreases over the course of HIV infection for reasons that are as yet unknown. Very recently, it has been shown that the memory/naive ratio is altered even in uninfected newborns of

HIV-infected mothers, suggesting that CMI and T-cell maturation may be altered even in HIV-uninfected newborns of HIV-infected mothers (Clerici et al., 2000).

The presence of both naive and memory T cells is associated with the condition of health. Thus, although naive T cells are important for their ability to respond to newly encountered pathogens, the presence of memory T cells is critically important in allowing the development of secondary immune responses. Recent data show that the number of memory $CD4^+$ T cells is increased much more promptly than that of naive T lymphocytes in HIV-infected individuals undergoing HAART (Autran et al., 1997; Gray et al., 1998; Wendland et al., 1999). Thus, whereas increases in $CD4^+45RO$ T cells are observed within a few months after the initiation of combination therapy, changes in the number of naive T lymphocytes are not detected for years in HIV patients undergoing HAART. This situation is characteristic of adult patients treated with HAART, as the number of naive T cells is promptly restored in HAART-treated pediatric patients (Vigano et al., 1999a). The ability of pediatric patients to repopulate peripheral blood with naive $CD4^+$ T cells upon the initiation of therapy was recently shown to be correlated with changes in the volume of thymus (Vigano et al., 2000) (Table 3.2).

CD25

CD25 is the α subunit of the IL-2 receptor. CD25 is expressed on activated T lymphocytes, where it forms a trimeric complex with the β and γ subunits of the IL-2 receptor. The trimeric form of this receptor binds to IL-2 in a much tighter way, thus increasing the affinity of the signaling receptor for IL-2. The percentage of $CD4^+$ T cells expressing CD25 is augmented in HIV infection; this increase is suggested to be directly correlated with increasing disease severity (Mahalingham et al., 1993, 1995). Interestingly, $CD4^+/CD25^+$ T lymphocytes are augmented even in HIV-infected individuals undergoing HAART. HAART-induced modulation of CD25-expressing $CD4^+$ T cells might be associated with the observation that, whereas suppression of HIV viremia can be achieved in the majority of HIV-infected individuals by antiretroviral therapy, the efficacy of these therapies in reconstituting immune function is less than dramatic. Thus, recent studies have suggested that CD25-expressing $CD4^+$ T cells are anergic suppressor cells (Thornton and Shevac, 1998, 2000) that can anergize $CD8^+$ T lymphocytes (Shimizu et al., 1999) and impair CMI via the production of IL-10 (Papiernick et al., 1998). An additional mechanism by which $CD4^+CD25^+$ T cells can dampen T-cell activation was recently postulated by Cederbom and colleagues, who showed these lymphocytes to be able to down-regulate the expression of CD80 and CD86 on the surface of antigen-presenting cells (Cederbom et al., 2000). Finally, the expression of CD25 was suggested to differentiate $CD4^+$ T cells that are latently ($CD25^-$) or productively ($CD25^+$) infected (Borvak et al., 1995).

TABLE 3.2. Correlation Between Changes in Thymus Volume and T-Cell Subsets After 12 Months of Potent Antiretroviral Therapy

CD28

CD28 is a disulfide-linked homodimeric glycoprotein that is expressed on the majority of human $CD4^+$ T cells and in 50% of CD8 T cells. CD28 expression augments after T-cell stimulation. CD28 binds to CD80 and CD86 on the surface of antigen-presenting cells; the interaction between these molecules delivers signals that augment T-cell responses to antigens. $CD8^+CD28^+$ T lymphocytes were suggested to have a pivotal role in the control of the progression of HIV infection as these cells have been shown to produce cell-antiviral factor (CAF), a soluble factor that can suppress HIV replication by blocking the transcription of viral RNA (Barker et al., 1997, 1998). The production of CAF diminishes in the progression of HIV disease (Blackbourn et al., 1996); an expansion of CD4 and CD8 T lymphocytes that do not express CD28 is observed as well in progressing patients (Kammerer et al., 1996). A tight correlation between the ex-

pansion of $CD8^+CD28^-$, $CD8^+CD38^+$, and $CD8^+CD45R0^+$ was also shown to be present in HIV-infected patients enrolled in a longitudinal study; these changes were suggested to be at least partially responsible for the functional defects of CMI that are characteristic of HIV infection (Choremi-Papadadopoulou et al., 1994). Of interest in this context is the observation that $CD8^+CD28^-$ T cells were described to function as T-suppressor lymphocytes that can inhibit the proliferative response of antigen-stimulated $CD4^+$ T-helper cells. Finally, $CD4^+CD28^+$ T lymphocytes were recently shown to be expanded in HAART-treated, HIV-infected individuals, and the percentage of $CD4^-$ and CD8-expressing CD28 T cells was demonstrated to be directly correlated with the rate of first HIV decay in patients upon initiation of antiretroviral therapy (Wu et al., 1999).

CD38

CD38 is a single-chain glycoproteic molecule that catalyzes the formation of cyclic adenosine diphosphate ribosomes from reduced nicotinamide adenine dinucleotide (Ferrero and Malavasi, 1997). CD38 is functionally a 45-kD type-II cell surface molecule involved in lymphocyte homing and, in particular, in the regulation of lymphocyte adhesion to endothelial cells (Deaglio et al., 1998). CD38 is also expressed in various human tissues including skeletal and cardiac muscle fibers, epithelial cells of bronchial origin, and cells from the parotis and hepatic sinusoids (Fernandez et al., 1999). The receptor for CD38 on the surface of endothelial cells was recently identified in CD31, a member of the immunoglobulin gene superfamily, and the interaction between CD31 and CD38 was shown to stimulate intracellular calcium fluxes and the synthesis of mRNA for an array of cytokines (Deaglio et al., 1998). CD38 is present in immune cells during hemopoiesis and disappears in resting, mature lymphocytes; expression of CD38 in these mature cells is subsequent to activation (Ferrero and Malavasi, 1997).

Analysis of CD38 expression on lymphocytes has become an important tool for monitoring patients during HIV-1 infection (Giorgi and Janossy, 1994; Giorgi et al., 1993, 1994) and has recently been proposed in the follow-up of HAART-treated individuals. The prognostic value of such analyses is attributed to the ability of CD38 to closely reflect the activation status of the immune response. The T-cell subset best investigated for CD38 expression is $CD8^+$. CD38 expression on $CD8^+$ T cells is up-regulated at seroconversion and is subsequently either maintained at a high level by individuals who rapidly progress to AIDS, or is partially decreased in subjects in the asymptomatic phase as compared with healthy controls (Giorgi and Janossy, 1994; Giorgi et al., 1993, 1994; Lenkei et al., 1998). Data from different studies indicate not only that the detection of a high proportion of $CD8^+CD38^+$ T cells predicts progression to AIDS in HIV-infected adults, but that the prognostic value of this marker is synergic to that of $CD4^+$ T-cell counts (Bofill et al., 1996; Lenkei et al., 1998). These findings have been attributed to the ability of CD38 to mark

activation of the immune system in possible correlation with disease progression. Moreover, some of the $CD8^+CD38^+$ lymphocytes display cytotoxic activity against viral antigen-expressing $CD4^+$ T cells, possibly contributing to $CD4^+$ T-cell depletion (Ho et al., 1993). More recently, other studies have shown that the presence of a high proportion of $CD8^+CD38^+$ T cells predicts the subsequent decline of $CD4^+$ T-cell counts (Bofill et al., 1996). Additionally, cross-sectional studies showed that $CD8^+CD38^+$ T cells correlate with increased titers of HIV plasma viremia in subjects treated with HAART (Burgisser et al., 1999), and to lack of response to therapy in children who are enrolled in antiviral protocols (Vigano et al., 1998). The prognostic value of CD38 expression on $CD8^+$ may be further improved by evaluating the co-expression on these cells of CD45RO. Thus, the relative proportion of $CD8^+CD45RO^+CD38^+$ T cells is predictive of CD4 cell decline, whereas the percentage of the $CD8^+CD45RA^+CD38^+$ population is not (Kestens et al., 1994). These findings may be explained by the observation that $CD8^+CD45RA^+$ T cells constitutively express CD38 even in normal controls and thus offer no significant contribution to the prognosis. On the contrary, $CD8^+CD45RO^+$ T cells express CD38 only upon cell activation, therefore rendering CD38 a good marker in evaluating activated T cells within the $CD45RO^+$ subset. In addition to expressing $CD45RO^+CD38^+$, the majority of $CD8^+$ antigen-specific T cells of HIV-infected individuals express major histocompatibility complex (MHC) class II antigens (Ho et al., 1994). Analysis of sorted populations of $CD8^+$ cells defined by HLA-DR and CD38 expression showed that the population that expressed both HLA-DR and CD38 had the strongest CTL activity, the HLA-DR^+ $CD38^-$ and HLA-DR^- $CD38^+$ populations had intermediate CTL activity, and, finally, the double-negative cells had very little activity (Ho et al., 1994). Furthermore, in HIV infection, the percentage of $CD8^+CD38^+$ T cells was observed to correlate with the defective proliferative response of mitogen-stimulated T cells. The expression of CD38 on $CD4^+$ T cells has been analyzed less thoroughly, but CD38 can also be up-regulated in this lymphocyte subset (Benito et al., 1997). $CD4^+CD38^+$ cells were found to rarely be increased during primary HIV-1 infection whereas this subset can be upregulated during the chronic phases of the disease. These cells were shown to mostly express HLA-DR in addition to CD38. This increase in CD38 expression correlates with the decline in $CD4^+$ T cells (Benito et al., 1997). It has also been proposed that the increase in the percentage of $CD4^+$ T cells that express CD38 at the late stages of the disease is a predictor of death.

CD95 (Fas)

CD95 (Fas antigen) is a protein expressed on the surface of lymphocytes (Miyawaki et al., 1992). Activation of Fas by its ligand (FasL) can activate T-cell proliferation and cytokine production or can induce apoptosis in susceptible cells (Suda et al., 1993). This system is finely regulated, as it was shown that a soluble form of Fas (sAPO-1/Fas) can prevent the interaction between

TABLE 3.3. Alterations of the Fas/Fas Ligand (FasL) and Soluble APO-1/Fas (sAPO-1/Fas) System in HIV Infection

- Up-regulation of Fas on lymphocytes of HIV-infected individuals
- Augmented susceptibility of lymphocyte of HIV-infected individuals to apoptosis upon ligation of Fas
- Correlation between expression of Fas/susceptibility of Fas ligation to apoptosis and clinical stage of HIV infection
- Monocyte-CD4/Fas-FasL "kiss of death"
- CD4-CD8/Fas-FasL "kiss of death"
- Reduced serum concentration of sAPO-1/Fas in progressing HIV infection

Fas and FasL thus reducing apoptosis (Cheng et al., 1994). CD95 and its ligand are expressed at high levels on activated mature lymphocytes, suggesting that CD95 expression and subsequent cell death may be the physiological consequence of cellular activation. Surface expression of CD95 is increased on lymphocytes of HIV-infected individual (Debatin et al., 1994; McCloskey et al., 1995). Additionally, the interaction of CD95 with its ligand in HIV-infected cells provokes apoptosis in a high percentage of lymphocytes (Sloan et al., 1997). Up-regulation of CD95 on monocytes is observed as well in HIV infection; the augmented expression of CD95 on these cells could be responsible for the destruction of uninfected CD4 T lymphocytes subsequent to antigenic presentation and interaction between CD95 (on monocytes) and CD95L (on CD4 cells), via a "kiss of death" mechanism (Badley et al., 1997).

Different experimental findings support a role for the quantitative/qualitative alterations of the CD95/CD95L system in the progression of HIV infection to AIDS (Table 3.3). These findings include the following:

1. Surface expression of CD95 is augmented in disease progression, and cells of individuals with AIDS and opportunistic infections express significantly more CD95 than asymptomatic patients (Debatin et al., 1994; McCloskey et al., 1995).
2. Apoptosis induced by engagement of CD95 on the surface of CD4 lymphocytes is higher in symptomatic than in asymptomatic HIV-infected individuals (Baulmer et al., 1996; Soland et al., 1997).
3. The magnitude of anti-CD95-induced apoptosis inversely correlates with absolute CD4 cell counts (Bohler et al., 1997a; Soland et al., 1997).
4. Low serum concentrations of sAPO-1/Fas were shown to have a strong independent predictive power for progression to AIDS in a multivariate conditional logistic regression model that included, among other variables, HIV viral load at entry (Medrano et al., 1998).

Another set of results underlines the importance of the CD95/CD95L system in the progression of HIV infection. Thus, T-helper 1 (TH1) but not TH2

undergo rapid CD95-mediated apoptosis upon antigen stimulation. TH2 clones are also known preferentially survive in vitro cell cultures. These findings are justified by the observation that, whereas both types of clones express CD95, only TH2 clones express high levels of a CD95-associated phosphate that inhibits CD95 signaling. Interestingly, a potent expression of CD95 as well as susceptibility to CD95 ligation seem to be predominant in primed/memory (CD45RO) T cells, suggesting that virus-driven T-cell activation is responsible for the increased apoptosis (Bohler et al., 1997b). This is also supported by findings of a prompt down-regulation of increased CD95L expression in T cells of patients undergoing antiretroviral therapy (Bohler et al., 1997c). This decrease is associated with reductions in HIV plasma viral load.

In addition to mechanisms of accelerated suicide or paracrine death, CD95-induced apoptosis during HIV infection may be mediated by cytotoxic effector cells. The magnitude of the CTL response during HIV infection and the high proportion of $CD95^+$ compliant target cells among patients' lymphocytes argue for a deleterious role of CTL against activated T cells. In the contest of HIV pathogenesis, the persistence of continuously activated antiviral CTL would thus be associated with CD95L expression and, therefore, with the ability to kill both virus-infected cells and noninfected, activated compliant $CD95^+$ target cells. The exacerbation of the CD95 system would drive antiviral CTL in a deleterious role and particulary in the physiological elimination of CD4 T cells, irrespective of the infected status. This would contribute to the collapse of the immune system.

PHENOTYPIC MARKERS PREFERENTIALLY EXPRESSED ON TH1 AND TH2 LYMPHOCYTES

Whereas TH1 and TH2 lymphocytes are still defined on a functional basis (i.e., the predominant cytokine production profile), a series of phenotypic markers may be preferentially expressed by either of the T-helper types. These markers include CD7, CD30, and some chemokine receptors (CXCR3, CCR3, CCR4, and CCR8) (see Table 3.4).

CD7

$CD4^+$ T-helper lymphocytes can be differentiated in two subpopulations based on the expression of the CD7 marker. $CD4^+CD7^+$ T lymphocytes preferen-

TABLE 3.4. Phenotypic Markers Preferentially Expressed by TH1 or TH2 Lymphocytes

TH1 lymphocytes	CD7*	SLAM	CCR3	CCR4	CCR8
TH2 lymphocytes	CD30	CXCR3			

*On $CD4^+CD57^+$ T cells.

tially produce IL-2 (in the absence of IL-4 and IL-10), whereas $CD4^+CD7^-$ T lymphocytes produce low amounts of IL-2 and high levels of the type-2 cytokines IL-4 and IL-10; additionally, lack of CD7 expression correlates with the acquisition of CD57 on $CD4^+$ T lymphocytes (Autran et al., 1995; Legac et al., 1992). The percentage of $CD4^+CD7^-CD57^+$ T lymphocytes proportionally increases in the progression of HIV infection to AIDS (Autran et al., 1995; Legac et al., 1992). The percentage of $CD4^+CD7^-CD57^+$ lymphocytes was also shown to be significantly increased when HIV-infected patients with progressive infection were compared with long-term nonprogressing individuals (Clerici et al., 1996b).

CD30

CD30 is a member of the tumor necrosis factor (TNF) receptor superfamily, which can transduce signals for proliferation, death, or nuclear factor KB (NF-KB) activation, and whose ligand (CD30L) has been identified on B cells, activated macrophages, and a subset of activated T cells. Expression of CD30 is augmented in a number of pathologic conditions including anaplastic large cell lymphomas, seminoma, and primary cutaneous lymphoproliferative disorders (Ansieau et al., 1996; Romagnani, 1996). Plasma levels of CD30 are significantly higher in HIV-1-infected patients than in controls, and are positively correlated with those of TNF-α and soluble TNF receptors as well as with HIV plasma viremia (Rizzardi et al., 1996). Additionally, in vitro HIV infection of $CD4^+$ T-cell clones generated from HIV-seronegative individuals can enhance the expression of CD30. TH2 cells preferentially express membrane CD30 and release the soluble form of CD30; additionally, CD30-mediated signaling was shown to promote the in vitro development of TH2-like cells (Romagnani, 1996).

Chemokine Receptors

Chemokines and their receptors are essential elements in determining the selective attraction of various cellular subsets of leukocytes. Two such receptors (CCR5 and CXCR4) have gained a pivotal role in the pathogenesis of HIV infection as they have been identified as the main coreceptors allowing the intracellular penetration of (respectively) monocytotropic and lymphotropic strains of HIV into target cells (Berger et al., 1999; Garzino-Demo et al., 1998; Loetscher et al., 2000). Polarized TH1 and TH2 cells were recently shown to differentially express chemokine receptors. Thus, the expression of CCR4 and CCR8 on TH2 cells was observed to be transiently increased following TCR and CD28 engagement; additionally, the response of TH2 cells to I-309 (CCR8 ligand) and thymus- and activation-regulated chemokine (TARC) (CCR4 and CCR8 ligand) was shown to be enhanced (Dambrosio et al., 1998). Other results showed that 1) naive T cells express CXCR4, whereas the majority of memory/activated T cells express CXCR3; 2) TH1-polarized T-cell lines ex-

press high levels of CXCR3, whereas TH2 express CCR3 and CCR4; and 3) only TH2 clones respond with an increase in $[Ca^{2+}]$ to the CCR3 and CCR4 agonists eotaxin and TARC, whereas only TH1 clones responded to the CXCR3 agonist IFN-γ-inducible protein 10 (Sallusto et al., 1998).

SLAM

SLAM is a glycoprotein of the Ig superfamily which, as a transmembrane lymphocytic receptor, is constitutively expressed on $CD45RO^+$ memory T cells, a portion of B lymphocytes, and immature thymocytes, and is also rapidly expressed on naive T and B cells after activation (Aversa et al., 1997; Cocks et al., 1995). SLAM engagement augments T-cell expansion and INF-γ production independently of CD28, and its signaling is regulated by the SLAM-associated protein (SAP). SLAM was observed to be up-regulated in human pathologies including rheumatoid arthritis and multiple sclerosis (Aversa et al., 1997; Cocks et al., 1995). Recent data have shown that 1) SLAM-expressing $CD4^+$ and $CD8^+$ lymphocytes are diminished in HIV infection; 2) SLAM expression on $CD4^+$ lymphocytes of HIV-infected individuals is preferentially associated with lack of CD7 ($CD4^+CD7^-SLAM^+$); and 3) SLAM engagement augments IFN-γ and reduces IL-10 production by HIV-stimulated PBMC of HIV-infected individuals (Meroni et al., 1999).

ANTIVIRAL AND IMMUNE MODULATORY EFFECTS OF HAART THERAPY IN HIV INFECTION

The current HAART, which usually includes a combination of antiretroviral compounds active on different viral enzymes, has modified the natural history of HIV infection. Although none of these compounds are very effective alone, combinations of two or more drugs have boosted optimism that HIV replication and disease progression can be controlled. It is nevertheless essential to underline that HAART is not the cure for HIV infection and that unsolved virologic, immunologic, and clinical problems are presented by HAART-treated individuals (Table 3.5). Particularly effective in controlling the progression of HIV infection are therapies based on the combination of reverse transcriptase and protease inhibitors (De Jong et al., 1998; Flexner, 1998). In this case, HIV replication will be impeded by two different mechanisms that will obstruct the action of two diverse viral enzymes (Richman, 1996). Viral replication will be potently suppressed as a consequence of these therapies and the emergence of multiple-resistant viruses will be delayed. Soon after initiation of HAART therapy, a string of dramatic effects on virologic and immunologic parameters are observed in the majority of patients (Angel et al., 1998; Kelleher et al., 1996, 1997; Martinon et al., 1999; Pakker et al., 1997, 1998; Pontesilli et al., 1999). First of all, it is possible to observe an increase in CD4 cell count. It is believed that the immediate increase in CD4 cell count that occurs during the

TABLE 3.5. Some Questions and Problems Stemming from HAART Therapy

Virologic problems
- How low should viremia be to prevent disease progression? Is it necessary to maintain viremia under the detection limit (>20 copies/mL)? Is it necessary to eradicate?
- How long will the suppression of HIV replication last?
- How can we reach HIV that is in latently infected cells and sheltered in particular anatomical sites (e.g., the central nervous system)

Immunologic problems
- Augmentation of CD4 T cells is partial and (probably) limited in time
- Augmentation of CD4 T cells is mostly supported by memory CD4 T cells
- Increase of CD4 T-cell functions is partial and HIV-specific functions are not recovered
- CTL are defective in HAART-treated patients

Clinical problems
- Access to therapy is not available to the vast majority of patients worldwide
- Lack of response to HAART in a sizable quantity of patients (HIV viral load is not suppressed and/or CD4 counts do not increase)
- Side effects
- Lack of availability of immune modulators

first several days of therapy is the result of memory cells re-entering the blood from lymphoid tissue (redistribution), together with a decrease in activation-induced apoptosis. Peripheral expansion (cell division), which is believed to occur slowly and continually, is only observed in the following weeks by a slower increase of CD4 cells once redistribution has occurred. However, many believe that the increase is caused by expansion alone. At present, the overall theory of the kinetics of T-cell reconstitution is under debate.

Other positive effects induced by HAART can be summarized as follows: 1) consistent and significant clinical improvements; 2) reduction in plasma HIV viral load; 3) limited increases in CD4 T-helper functions (antigen-stimulated proliferation, cytokine production); and 4) reduction in the percentage of activation markers bearing cells (i.e., diminished expression of CD38 on $CD8^+$ T cells).

Nevertheless, HAART is ineffective in a sizable percentage of patients (up to 50% of individuals fail first-line therapy and up to 80% fail second-line rescue therapy) (Kaufman et al., 1998). HAART unresponsiveness can be manifested as a lack of virologic effect (i.e., HIV plasma viral load does not diminish significantly), a lack of immunologic effect (CD4 counts do not improve), or as a lack of both virologic and immunologic effects) (Kaufman et al., 1998). Recent data show that virological failure (i.e., significant increases of HIV viral load after an initial reduction of viremia) is a relatively common phenomenon in HAART-treated individuals, and that salvage therapy in these individuals is rarely efficacious. Prompt identification of HAART-unresponsive patients

would allow initiation of alternative, and hopefully more successful, drug regimens. This necessity has stimulated in-depth analyses finalized at the definition of possible prognostic markers capable of predicting which patients will more likely be therapeutic failures. For example, consistent with their negative prognostic value, an elevated percentage of $CD8^+CD38^+$ T lymphocytes might predict unresponsiveness to HAART. The biologic basis for negative prognostic value of $CD8^+CD38^+$ T cells is unknown; different hypotheses have been put forward to explain these puzzling observations. To summarize, augmented percentage and/or expression of $CD8/CD38^+$ could be a poor prognostic factor in HIV infection because 1) $CD8^+CD38^+$ T cells mediate cytotoxicity directed toward autologous HIV-infected and uninfected CD4 T cells; 2) $CD8^+CD38^+$ T cells are immature T cells, thus indicating the abnormality of the immune system and possible alterations in hemopoiesis; 3) $CD8^+CD38^+$ T cells might be poor producers of antiviral factors; and 4) $CD8^+CD38^+$ T cells might be the immune correlate of response to high loads of virus.

Because augmented percentages of $CD8^+CD38^+$ T cells could be an early predictor of treatment failure, particular attention should be given to those individuals in whom an abnormal augmentation of this subset is detected. Finally, it must be underlined that the effects of HAART in reconstituting the functionality of the immune response (e.g., antigen-stimulated proliferation and cytokine production) is still less than optimal (Clerici et al., 2000; Martinon et al., 1999). This observation strongly supports the concept that immune modulation has to be considered an essential component of the therapeutic approach to HIV infection.

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4

Quantification of HIV/SIV Coreceptor Expression

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INTRODUCTION

Multiparametric fluorescence-activated cell sorter (FACS) analysis, including the use of recently developed quantitative approaches, is assuming great importance in the study of human immunodeficiency virus (HIV)-1 infection as a result of the fact that cellular entry by HIV-1 requires the sequential interaction of the viral envelope protein (Env) with cell-surface CD4 and a coreceptor. CCR5 and CXCR4, members of the chemokine receptor family, have been identified as the major coreceptors for viral entry (reviewed in Berger et al., 1999; Doms and Peiper, 1997; Lee and Montaner, 1999). All HIV-1 isolates studied to date use either one or both of these chemokine receptors for viral entry (Doms et al., 1998). In general, macrophage-tropic strains of virus, predominantly isolated during the early stages of HIV infection and implicated in sexual transmission of the virus, use CCR5 as a coreceptor. On the other hand, T-cell tropic strains of virus, either T-cell line lab-adapted strains or those that are more frequently isolated during progression to clinical disease, use CXCR4 as a coreceptor (reviewed in Husman and Schuitemaker, 1998). A significant minority of primary patient isolates is also able to use both CCR5 and CXCR4 as coreceptors. The importance of coreceptor usage for the paradigm of HIV tropism is underscored by the new terminology in which viral strains are now

designated by the coreceptor(s) that they use for cellular entry. Hence, viruses that use either CCR5, CXCR4, or both for entry are now designated R5, X4, or R5/X4 viruses, respectively (Berger et al., 1998).

CCR5 and CXCR4 also serve as the major coreceptors for HIV-2, whereas CCR5 is the major coreceptor for simian immunodeficiency virus (SIV) (Doms et al., 1998). Other members of the chemokine receptor family or even other G-protein coupled receptors (GPCRs) have been ascribed coreceptor activity in vitro, at least for select viral strains (Choe et al., 1996, 1998; Deng et al., 1997; Doranz et al., 1996; Edinger et al., 1998; Farzan et al., 1997; Horuk et al., 1998; Liao et al., 1997; McKnight et al., 1998; Reeves et al., 1997; Rucker et al., 1997; Samson et al., 1998; Shimizu et al., 2000). However, the in vivo significance of these alternate coreceptors remains obscure, as the vast majority of these isolates are unable to replicate in PBMCs in the presence of CCR5- and CXCR4-specific antagonists (Zhang et al., 2000). The paramount importance of CCR5 in the pathogenesis of acquired immunodeficiency syndrome (AIDS) is indicated by genetic-epidemiological evidence that individuals who are homozygous for a CCR5-null mutation (*ccr5Δ32*) are highly resistant to HIV infection, whereas HIV-infected individuals who are heterozygous for *ccr5Δ32* progress to clinical AIDS more slowly than their homozygous CCR5 wild-type counterparts (reviewed in Carrington et al., 1999), most likely because of a relatively modest reduction in CCR5 expression (Wu et al., 1997). Indeed, coreceptor expression levels can have a significant impact on viral infectivity (Platt et al., 1988; Sharron et al., 2000). The presence of a disease progression modifying allele in the SDF-1 gene (Balotta et al., 1999; Brambilla et al., 2000; John et al., 2000; Mummidi et al., 1998; van Rij et al., 1998; Winkler et al., 1998), which codes for the natural chemokine ligand to CXCR4, coupled with the increased pathogenicity of CXCR4-using strains (Schramm et al., 2000) predominantly isolated from patients in later stages of HIV disease also points to the in vivo importance of CXCR4 in HIV pathogenesis.

The use of CD4 and various coreceptors by the primate immunodeficiency viruses to enter cells indicates that the expression patterns of these receptors is of critical importance in governing viral tropism: the ability of a virus strain to infect some cell types but not others. In addition, because the membrane fusion process mediated by the viral Env protein is highly cooperative in nature, requiring both multiple Env proteins as well as multiple receptor interactions to form a fusion pore (Kuhmann et al., 2000), it logically follows that expression levels of the various receptors will also influence viral infectivity. As a result, multiparametric FACS analysis of CD4 and coreceptor expression is ideally suited for studies designed to identify which of the myriad T-cell subsets are susceptible to infection by any given virus strain. The introduction of rigorous, quantitative FACS techniques, often in conjunction with multiparametric analyses, has made it possible to identify not just the cell types that express the receptors needed to support virus infection, but to identify cells that express these receptors at sufficiently high levels. These approaches are also needed to dissect the web of cytokine and immune system interactions that influence re-

ceptor expression, and hence viral infectivity. In this chapter, we will review how the expression patterns of CCR5 and CXCR4 impact HIV pathogenesis and point out examples of how multiparametric FACS analysis of coreceptor expression has provided important information on HIV-receptor interactions. In addition, investigations into the efficiency of coreceptor usage, which may impact the pathogenicity of the viral isolate in question, also demands the ability to quantify the number of coreceptor molecules on the cell surface. We will review the use of quantitative FACS as a way to put absolute numbers on the levels of coreceptor expression and argue that future studies involving coreceptor expression levels adopt this methodology so that results from varying groups can be more readily compared.

EXPRESSION PATTERN AND LEVELS OF CORECEPTOR HAVE IMPLICATIONS FOR HIV PATHOGENESIS

Early in the HIV/AIDS epidemic, the level of circulating CD4-positive T cells provided the most easily quantifiable parameter that could be used to measure disease progression. Although still providing important information on immune status, measurements of virus load (the number of viral RNA molecules per milliliter of blood) are now the best predictor of disease course. Similarly, it is now apparent that simple measurement of CD4 levels per se is overly simplistic, inasmuch as HIV requires both CD4 and an appropriate coreceptor for infection. The differential expression of CCR5 and CXCR4 on immune subsets clearly impacts the type of cells that are susceptible to the pathogenic consequences of viral infection.

The first indication that CCR5 and CXCR4 are differentially expressed on subsets of $CD4^+$ lymphocytes comes from studies by Bleul et al. (1997) showing that CCR5 expression is largely restricted to $CD45RO^+$ memory subsets whereas CXCR4 expression is more skewed toward $CD45RA^+$ naive subsets. These observations were subsequently confirmed by others (Lee et al., 1999b; Mo et al., 1998) and substantiated by evidence showing that, although R5-using viruses mainly infect memory $CD45RO^+$ cells in vivo and in vitro, infection of $CD45RA^+$ naive T cells is usually via syncytium-inducing, X4-using viruses (Blaak et al., 2000; Ostrowski et al., 1999). The preferential infection of memory cells in vivo by both X4 and R5 viruses and of naive cells by X4-using viruses closely parallels the differential cell surface expression of CCR5 and CXCR4 (Blaak et al., 2000). Importantly, the frequency of X4 virus-infected $CD45RA^+$ naive cells correlates with $CD4^+$ T-cell decline (Blaak et al., 2000), giving an example of how multiparametric FACS analysis of coreceptor expression in the context of immune system markers may shed light on the immunopathogenesis of AIDS.

Although coreceptor expression levels are an important variable governing viral infectivity, expression levels alone do not necessarily predict the extent of viral replication in vivo. For example, acute activation of peripheral blood

lymphocytes (PBLs) in patients undergoing interleukin (IL)-2 immunotherapy led to a dramatic increase in the number of CCR5-expressing PBLs with no accompanying increase in viral load (Weissman et al., 2000; Zou et al., 1999). This apparent incongruence may potentially be explained by separate observations that inhibitory CC-chemokines are a predominant product of activated $CD45RO^+$ memory phenotypes, particularly within the interferon (IFN-)γ secreting subset (type-1 cells), precisely the subset that would be activated by IL-2 treatment. Multiparametric FACS analysis has the potential to clarify this issue by combining intracellular cytokine staining with careful examination of chemokine receptor expression in the subset involved.

It is important to realize that although in vitro experiments indicate that threshold levels of CD4 and coreceptor are required for efficient infection, susceptibility of primary cells in infection in vivo is dependent not only on the level of coreceptor expression but also on the level of cognate chemokine production. Coreceptor expression on T-helper subsets is polarized with CCR5 predominantly expressed on type-1 T-helper (TH1) cells and CXCR4 expression skewed toward the TH2 subset. At first sight, the high level of CCR5 expression on TH1 cells would be expected to render these cells highly susceptible to infection by R5 viral strains were it not for modulating effects of endogenously produced beta-chemokines (Annunziato et al., 2000). Similarly, the extraordinary amounts of ''Regulated upon Activation, Normal T cell expressed and secreted'' secreted by megakaryocytes have been proposed as an explanation as to why these cells are resistant to R5 but not X4 viruses despite the expression of CD4 and both coreceptors (Chelucci et al., 1998; Majka et al., 1999; Park et al., 1999). In addition, the constitutive secretion of SDF-1 by mucousal epithelial cells may partially account for the preferential transmission of R5 viral strains across mucosal barriers despite the ample presence of $CD4^+/CXCR4^+$ lymphocytes (Agace et al., 2000). Thus, although CD4 and coreceptor expression render a cell potentially susceptible to HIV infection, the requirements for viral entry and replication are certainly more complicated than the physical presence of these viral receptors. External factors such as inhibitory chemokines and post-entry factors that allow for a permissive intracellular milieu should be taken into account, but FACS analysis of coreceptor expression is an important component in the calculus of factors responsible for viral entry and replication.

COMPLEXITIES OF CORECEPTOR EXPRESSION REQUIRE CAREFUL QUANTITATIVE ANALYSIS OF EXPRESSION PATTERNS

The factors affecting chemokine receptor expression are legion and are beyond the scope of this chapter (reviewed in Lee and Montaner, 1999; Murdoch and Finn, 2000; Ward and Westwick, 1998; Ward et al., 1998). However, the complexity of the network involved in regulating chemokine receptor expression on different subsets of immune cells presents a problem uniquely suited to multicolor FACS analysis. Unfortunately, the plethora of data regarding which fac-

tors up-regulate or down-regulate which coreceptor has generated a sense of confusion regarding not only the relevance of the observations seen but also in the reproducibility of the effects reported. Even reproducible effects such as IL-2's up-regulation of CCR5 on primary T cells is of questionable relevance for HIV-1 infection in vivo if such an increase in $CCR5^+$ cells does not lead to a detectable increase in viral load (Weissman et al., 2000). On the other hand, endogenously regulated levels of CCR5 in vivo appear to have an effect on viral replication as individuals heterozygous for the CCR5-null mutation, *ccr5Δ32*, clearly have a delayed progression to clinical AIDS (reviewed in Carrington et al., 1999) and appear to have lower viral loads than their wild-type counterparts, at least in some studies (Katzenstein et al., 1997; Walli et al., 1998). Furthermore, the panoply of CCR5 promoter polymorphisms with disease-modulating effects suggests that genetically determined responses of CCR5 expression levels to immunoregulatory effects can dictate how well individuals can support R5 viral replication. The problem remains that there has been no systematic attempt to quantify the levels of coreceptor affected by these genetic and immunoregulatory factors (see below for exceptions).

FACS analysis of coreceptor expression also presents technical difficulties not immediately obvious to the uninitiated. For example, Ficoll purification of lymphocytes acutely and dramatically down-regulates expression of some viral coreceptors (Lee et al., 1999a; Sharron et al., 2000; Weissman et al., 2000), obscuring attempts to correlate chemokine receptor expression with immunological or virologic parameters in vivo. In addition, chemokine receptors can exist in distinct conformational states, not all of which may be equally accessible to the virus (Lee et al., 1999a), making the choice of the antibody used an important variable. Recent advances in flow cytometry have suggested the importance of quantifying cell surface antigenic density in addition to the frequency of antigen-positive cells (Lavabre-Bertrand, 1996; Poncelet et al., 1996). Antigenic density of certain cell surface markers has been ascribed prognostic significance in some malignancies (Cabezudo et al., 1999; Kuss et al., 1999) and in HIV infection, increased antigenic density of the activation marker CD38 on $CD8^+$ T cells has been associated with poor prognosis (Liu et al., 1996). Given the complexities involved in measuring coreceptor expression, we propose that measuring the absolute antigenic density of coreceptors on relevant immune cells may allow for comparisons of data generated from various labs and provide a standardized format to compare the myriad immunoregulatory networks affecting coreceptor expression. Quantitative FACS analysis provides a way to measure absolute antigenic density.

QUANTITATIVE FACS (QFACS) ANALYSIS

Quantitative FACS analysis (QFACS) is based on the principle of converting the relative mean fluorescence intensities (MFI) of a particular analyte cell population into an absolute number of antibody binding sites (ABS). This

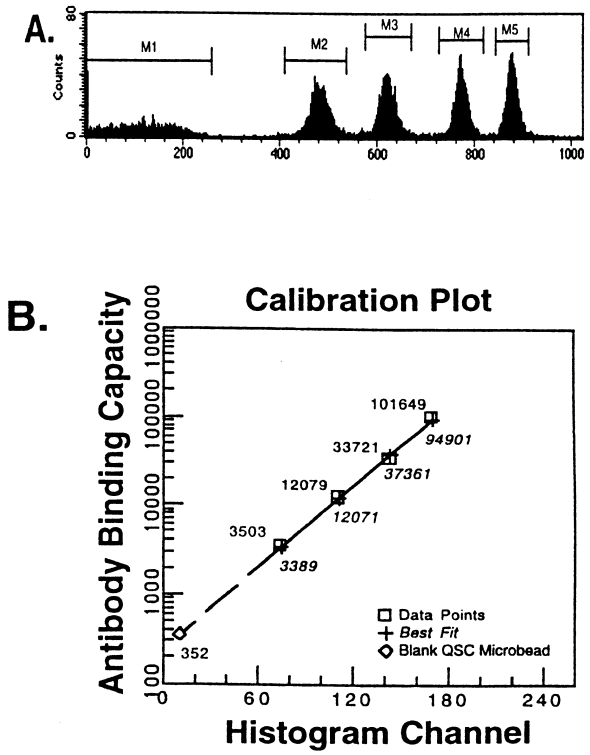

Figure 4.1. Quantitative flow cytometry (QFACS) techniques have been developed that use populations of highly uniform microbeads that bind specific amounts of fluorochrome-conjugated murine immunoglobulins (M1, M2, etc. peaks in **A**). The beads, approximately the size of human peripheral blood lymphocytes, are processed and analyzed in the same way as the experimental samples. A standard curve is generated using the peak channel fluorescence intensities of each bead population (staining of beads with anti-CD4-PE is shown in **A**, the resulting calibration curve is shown in **B**). Thus, it is possible to determine the number of antibody-binding sites on a particular cell population.

conversion can be done directly, by comparison to a standard curve generated by a series of microbeads predetermined to bind a fixed number of antibody molecules, or indirectly, by comparison to a standard curve generated by beads conjugated with differing levels of fluorochrome molecules. An example of the former is the Quantum Simply Cellular^{TM} (QSC) kit from Sigma (St. Louis, MO), whereas the QuantiBRITE^{TM} (QB) system (Becton Dickinson, San Jose, CA) is a popular example of the latter system, although many other companies make standardized fluorescence beads (e.g., Spherotech, Libertyville, IL; Molecular Probes, San Diego, CA). Figure 4.1 shows an example of a typical regression curve generated from the QSC system using a phycoerythrin (PE)-conjugated anti-CD4 antibody. The system consists of five microbead populations of uniform size coated with goat-anti-mouse antibodies: four populations bind an increasing number of mouse antibodies whereas one population does

not bind any and is used as an internal negative reference point to set the negative gate. After adding a saturating amount of the antibody to the beads and processing them identically to the samples being quantified, the MFI of each positive bead population is then linearly regressed against antibody binding capacities of the stained microbeads. The MFI of each antigen being analyzed on the sample cells can then be converted to ABS by comparison with the standard curve generated. In contrast to the QSC system, the QB system does not require prestaining because the beads provided come with a fixed number of PE molecules on each population. However, only PE-conjugated antibodies may be used with this system.

Although the general principles of QFACS are quite simple, meaningful quantification of ABS requires taking into account the many variables that may affect the validity of the regression curve generated and the relationship between the MFI of the sample antigen and the values on the regression curve. Information provided with each system indicates how to evaluate the standard curve to determine if staining and instrument conditions were adequate. A number of the more important variables are discussed below.

Fluorophores

PE has the highest Stokes shift and quantum efficiency among the common fluorophores used in FACS analysis and thus is ''brighter'' than the other fluorophores and can lead to higher sensitivities. More importantly, PE does not self-quench and thus can ensure the linearity of the regression curve at high densities, making it possible to accurately measure high-density cellular antigens. By contrast, dyes such as fluorescein isothiocyanate (FITC) can self-quench at higher densities (Haugland, 1996), presumably as a result of their greater overlap in excitation and emission spectra. Nontandem ''third'' color dyes such as PerCP, although not subject to self-quenching, are photolabile and degradation of the fluorophore while transiting through the laser beam of the cytometer makes careful quantitation difficult at best. Tandem third-color dyes such as Tricolor^{TM} or Cychrome^{TM} (PE-cy5) may be affected by inappropriate intermolecular fluorescence energy transfers at high antigen densities (intramolecular PE to Cy5 energy transfers are the only desirable ones), once again complicating the linear response required for accurate quantification. In addition, especially when using the QB system, it is essential that the fluorophore/antibody conjugate ratio be uniformly close to 1. Because of the large size of PE (240 kD), chemical conjugation to PE followed by high-performance liquid chromatography (HPLC) purification can generally lead to 100% pure 1:1 antibody/PE conjugates (this is also true for protein-chromophores like allophycocyanin). Conjugation to small molecular dyes such as FITC cannot be controlled to achieve such uniform labeling. Therefore, although standardized beads exist conjugated to many fluorophores, accurate quantitation of ABS using QFACS requires the use of PE (or other members of its class, e.g. allophycocyanin) for the reasons discussed. Finally, although the newer dyes such

as the Alexa series from Molecular Probes may have increased photostability and less self-quenching, it is still not possible to ensure a uniform conjugation ratio with these small molecular dyes.

Direct Versus Indirect Conversion

Calibration using the QSC system allows direct conversion of MFI into ABS per cell whereas the QB system converts MFI into the number of PE molecules bound per cell, again indicating the importance of using a fluorophore/antibody conjugate ratio close to 1. Purification of 1:1 conjugates is usually done by HPLC purification of PE-conjugated antibodies, using fractions that give a 565 λ/280 λ ratio of 3.4 ± 0.2. Most commercially available PE conjugates may contain mixtures of multimers (2:1 to 1:2) and 1:1 conjugates. The ABS value obtained for a specific antigen also depends on the valency of antibody-antigen interaction, which can only be determined experimentally. Another presupposition in using the QSC system is that the stoichiometry of binding at high antibody densities is the same on the beads as on the cell.

Reproducibility

Quantitative FACS analysis allows data to be compiled and compared from multiple experiments without having to rely on subjective interpretations of slight shifts in MFI. In our experience, using the Kolmogorov-Smimov (K-S) statistics to determine if slight shifts in histogram profiles are significant can be misleading; we have done experiments to show that two isotype controls when overlaid can sometimes give p values of <0.05 when using the K-S statistics profile on the CellQuest™ (Becton-Dickinson, San Jose, CA) program. However, QFACS relies on a strict set of predetermined parameters, which, when satisfied, allows one to place a degree of confidence in the numbers generated. When using QFACS, the above-mentioned isotype controls gave ABS values within the standard error stipulated by the regression curve generated and thus were not considered as significantly different.

Quantitation of cell surface antigens is only meaningful when results can be compared between laboratories. Therefore, for meaningful comparisons to be made, ABS values can only be compared when using the same monoclonal to the same cell surface antigen. Any extrapolation to the number of actual antigenic sites requires experimental determination of the valency of antibody binding.

QFACS ANALYSIS FOR CORECEPTOR EXPRESSION

For quantitative determination of coreceptor expression levels, it is crucial that the most appropriate antibody be used. There is increasing evidence that CCR5

and CXCR4 exhibit conformational heterogeneity and choosing the "wrong" antibody may affect the ABS value obtained by several fold (Lee et al., 1999a; and data not shown). For CCR5, 2D7 (Pharmingen, Torrey Pines, CA) appears to recognize the greatest number of cell surface CCR5 molecules of the antibodies we have tested (Lee et al., 1999a). For CXCR4, although the 12G5 clone has been used extensively for FACS analysis (primarily due to its early availability), it appears that clones 45701, 45717, and 45718 (R&D Systems, Minneapolis, MN) reproducibly recognize a larger number of CXCR4 molecules on primary peripheral blood lymphocytes. More importantly, these clones also appear to inhibit more completely CXCR4-mediated infection, implying that they bind most of the CXCR4 epitopes used by X4 viruses. Thus, although early studies on CXCR4 quantification have used the 12G5 clone, we recommend that future studies use the above-mentioned clones from R&D Systems.

Another important consideration in performing QFACS analysis for coreceptor expression is that chemokine receptors are particularly susceptible to down-regulation upon Ficoll purification and their levels are altered only lymphocytes even when sitting in anticoagulated whole blood left at room temperature (Reynes et al., 2000; Sharron et al., 2000; Weissman et al., 2000). In addition, there is great individual variability in the modulatory effects of in vitro culture conditions on CCR5 and CXCR4 expression on primary lymphocytes and macrophages. Therefore, studies attempting to correlate chemokine receptor expression levels with parameters of HIV disease progression should be performed on fastidiously fresh whole blood using the poststaining red blood cell lysis method. Indeed, the reproducibility of the QFACS assay is evidenced by the fact that three separate groups using the same monoclonal antibody (2D7 for CCR5) have arrived at the same value for the antigenic density of CCR5 on peripheral blood $CD4^+$ cells ($\sim$5,000–10,000 antibody binding sites per cell) (Hladik et al., 1999; Lee et al., 1999b; Reynes et al., 2000). This was true despite the fact that standardized beads from three different companies were used (QSC, Sigma, direct conversion; Qifikit, Dako, direct conversion; RCP-30-5, Spherotech, indirect conversion).

Given the reproducibility of the technique, we believe that QFACS should be applied to some of the problems relating to chemokine receptor expression in HIV pathogenesis. For example, it is now a well-established fact that cellular activation in vitro leads to increased CCR5 expression (de Roda Husman et al., 1999; Moore, 1997; Moriuchi et al., 1997; Ostrowski et al., 1998; Wu et al., 1997) but only recently has it been established quantitatively (via QFACS) that in vivo activation (as judged by human leukocyte antigen DR [HLA-DR] expression) also results in increased CCR5 expression (Reynes et al., 2000). However, markers of immune activation are heterogeneous and noncoincident; it would be fruitful for future studies to determine whether CCR5 and/or CXCR4 expression vary with other activation markers such as CD25, CD26, or even subsets of cytokine-secreting cells (IL-2 or IFN-γ).

In vitro studies indicate that R5 and X4 viral strains can have differential

requirements for CD4 and coreceptor levels (Kozak et al., 1997; Platt et al., 1998). Platt et al. (1998) determined that, where CD4 levels were "limiting" ($< 10^4$), levels of CCR5 below a threshold of $1–2 \times 10^4$ molecules significantly affected the efficiency of infection. However, when CD4 levels were high (4.5×10^5), minimal levels of CCR5 (2×10^3) supported maximal viral infectivity. In addition, primary X4 viruses appear more dependent on CD4 levels than lab-adapted strains (Kabat et al., 1994). Because CD4 antigen density on PBMC is quite high (between 10^4 and 10^5 molecules per cell (Lee et al., 1999b) whereas coreceptor levels are typically less than 10^4 per cell, it is likely that endogenous coreceptor levels (Hladik et al., 1999; Lee et al., 1999b; Reynes et al., 2000) will have a greater impact on the efficiency of viral entry. In addition to the slower disease progression noted in HIV-infected *ccr5Δ32* heterozygotes, presumably due to lower expression of CCR5 on lymphocytes and lower viral loads (see above), there is evidence to indicate that CCR5 expression on cell types other than immune cells may also impact on disease pathogenesis and survival. For example, one study showed an under-representation of *ccr5Δ32* genotype in patients with AIDS dementia complex (van Rij et al., 1999). Because CCR5 is required for viral replication in macrophages (Rana et al., 1997) and microglial cells in the central nervous system (Albright et al., 1999; He et al., 1997; Shieh et al., 1998), this suggests that expression levels of CCR5 can directly affect the size of a macrophage-tropic reservoir of HIV-1 in the brain. Indeed, we have quantified various chemokine receptors on microglial cells (CCR5, CCR3, and CXCR4) and found that, despite the ample presence of chemokine receptors with coreceptor activity, CCR5 is still the major coreceptor used in these cells (Albright et al., 1999). As such, this serves as another example whereby careful quantification of coreceptor expression has led to insights regarding specific aspects of the viral pathogenesis.

CONCLUSION

The identification of the cellular receptors used by HIV to infect cells has provided considerable insight into viral tropism and pathogenesis. However, full understanding of the complex interactions between the virus and target cells in vivo requires more accurate measurements of receptor density. Attempts to do so are complicated by the dynamic manner in which these receptors are regulated, the intricate network of chemokines and cytokines that can directly or indirectly affect receptor expression, and the fact that both CCR5 and CXCR4 exist in antigenically distinct conformations. The use of QFACS makes it possible to quantify receptor expression in an easy and reproducible manner, obviating many of the experimental difficulties encountered by earlier studies. By coupling this approach with virological and clinical data, it should be possible to more fully understand the complex interactions between the virus and its receptors.

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PART II

CELLS

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5

B Cells in the Line of Sight of HIV-1

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INTRODUCTION

Infection with human immunodeficiency virus (HIV)-1 results in a wide spectrum of clinical manifestations. HIV-1 preferentially infects $CD4^+$ cells including monocytes, T cells, and cells from the central nervous system (CNS), but the peripheral B cells of HIV^+ patients have an abnormal phenotype (Amadori and Chieco-Bianchi, 1990; Lane et al., 1983; Martinez-Maza et al., 1987) and spontaneously produce high levels of antibodies (Ab) (Amadori et al., 1989). Forster et al. recently reported a decrease in CXCR5 expression, a chemokine receptor important for the migration of B cells into lymphoid organs, on peripheral B cells from HIV^+ patients (Forster et al., 1997). Within lymphoid organs, HIV replication occurs mainly in areas essential for antigen-specific T-B interactions, and germinal centers (GC) are required for HIV-1 replication (Hufert et al., 1997; Pantaleo et al., 1995). Soon after HIV infection, strong follicular hyperplasia is detectable within the lymphoid organs of HIV^+ patients and persists throughout the asymptomatic phase, whereas it rapidly decreases during normal immune responses (Cohen et al., 1995; Racz et al., 1986). In addition to an increase in size and disorganization of the follicular dendritic cells (FDC) network, we have observed a thin and frequently disrupted mantle zone, a loss of GC polarization, and a decrease in CD80 expression on GC B cells of

hyperplastic HIV^+ lymph nodes (Legendre et al., 1998). Persistent HIV infection is accompanied by progressive and irreversible destruction of lymphoid organs involving not only $CD4^+$ T cells, but also B cells and FDC (Baroni and Uccini, 1990; Heath et al., 1995; Spiegel et al., 1992).

These data show that HIV-1 induces strong B-cell activation and the disorganization of lymphoid tissue. Besides impairing cell-cell interactions and cytokine production, we thought that these functional and phenotypic changes result from direct interactions between virions and B cells. We have therefore studied over the last few years the mechanisms by which HIV-1 alters the various steps of B-cell differentiation and may actively participate in lymphoid tissue disorganization and disease progression.

NORMAL B-CELL DIFFERENTIATION AND TRAFFICKING

In the absence of antigenic stimulation, mature B and T lymphocytes continuously pass from the blood into the lymphoid tissue. Contact with the antigen (Ag) that occurs in the epithelia of skin or mucosa induces the recirculation of immature dendritic cells (DC) into the draining lymphoid organ (Cyster, 1999). The binding and uptake of Ag result in the maturation of DC and their relocation to the junction between the extrafollicular zone of the lymphoid tissue, rich in T lymphocytes, and the external area of the follicles, rich in B lymphocytes (Cyster, 1999). At this site, Ag presented by mature DC independently activates B and T lymphocytes with the same antigenic specificity, preventing their further recirculation (Liu, 1997; McHeyzer-Williams et al., 1993; Melchers et al., 1999). An efficient T-dependent humoral response is only achieved if these Ag-activated B and T lymphocytes rapidly establish direct cell-cell interactions, allowing the B cells to survive, to differentiate into plasmocytes producing immunoglobulin (Ig)M with low affinity for the Ag, and to generate GC founder cells (Goodnow and Cyster, 1997; Liu, 1997; Melchers et al., 1999). Within GC, B lymphocytes are subjected to several cycles of a sequence of events: a clonal expansion phase during which VDJ hypermutations generate antibody variants (dark zone of GC, centroblasts) followed by a second phase involving the arrest of cell proliferation, isotype switching, and the selection of centrocytes expressing B-cell antigen receptor (BCR) with high affinity for the Ag (light zone of GC) (Berek et al., 1991; Kelsoe, 1996; Liu et al., 1992). Hypermutation and subsequent selection of variants that bind Ag strongly result in a progressive increase in Ab affinity, a process known as affinity maturation (French et al., 1989; Griffiths et al., 1984). Both negative and positive selection processes control affinity maturation. Negative selection, leading to a massive apoptosis, is favored by the down-regulation of Bcl-2 and the strong expression of Fas (CD95) in GC B cells (Martinez-Valdez et al., 1996; Smith et al., 1995). Several pathways may lead to GC B-cell apoptosis: BCR triggering in the absence of T-cell signals, ligation of CD95 by CD95L-

expressing T cells in the absence of potent BCR triggering, or asynchronous signaling through CD95, CD40, and BCR (Cleary et al., 1995). However, data obtained in genetically modified mice are not consistent with major roles for Bcl-2 and Fas in affinity maturation, but instead suggest that Bcl-2 facilitates the survival of autoreactive B cells in the periphery (Smith et al., 1994, 1995). In contrast, affinity maturation seems to be controlled by Bcl-$_{xL}$: *bcl-xl* transgene expression increases the frequency of B cells bearing VDJ rearrangements encoding low-affinity Ab, reduces the level of apoptosis in GC B cells, and facilitates a relaxed negative selection in GC (Takahashi et al., 1999). Affinity maturation is therefore reduced in *bcl-xl* transgenic mice. The crosslinking of BCR or CD40 extends the survival of centrocytes and up-regulates both Bcl-2 and Bcl-$_{xL}$ expression in mice and humans (Liu et al., 1989; Tuscano et al., 1996). Negative selection also takes place by receptor editing in centrocytes ($CD77^{-}$, $CD86^{high}$) re-expressing RAG-1 and RAG-2 proteins (Giachino et al., 1998; Han et al., 1996). Selected centrocytes then differentiate into Ag-specific memory B cells and long-lived plasmablasts, producing IgM, G, A, and E with a high affinity for Ag. Plasmablasts and memory B cells then leave the follicle, migrating to specific areas in which they complete their differentiation.

Accurate synchronization is required between the circulation of lymphoid cells and DC, the expression of molecules controlling cell-cell interactions or interactions with the extracellular matrix, the expression of cytokine receptors, and the production of cytokines to generate an efficient humoral response. Primary challenge with T-dependent Ag induces a strong GC response but a weak extrafollicular response, whereas secondary challenge leads to a strong extrafollicular response and a low GC response (Kroese et al., 1990; Liu et al., 1991). This reflects the location of the Ag targets: naive B cells giving rise to GC founder cells during primary responses and memory B cells located in the extrafollicular areas of lymphoid organs during secondary responses. It seems likely that memory B cells rapidly transit through GC, in which they are restimulated by Ag trapped on FDC. During the primary response, the size of the GC rapidly increases with the entry of GC founder cells and their subsequent clonal expansion, the expansion of the FDC network, and the entry of CXCR5-expressing $CD4^{+}$ cells (Forster et al., 1996). Recent data from studies of mice have clearly shown that the colonization and the functioning of the GC require interactions between B cells, FDC, and immune complexes bound to FDC (Ngo et al., 1999). The normal development and function of the FDC network depend on two major cytokines, lymphotoxin $\alpha 1\beta 2$ ($LT\alpha 1\beta 2$) and tumor necrosis factor-α (TNF-α), probably produced by Ag-triggered B cells within the GC (Futterer et al., 1998). Following Ag stimulation, the entry of B cells into GC requires the local production of a recently identified chemokine, B cell-attracting chemokine 1 (BCA-1), by FDC and DC (Cyster, 1999; Melchers et al., 1999). GC enlargement decreases after 3–4 weeks of Ag stimulation: B-cell expansion decreases, the effector B cells (long-lived plasmablasts or memory cells) leave the GC, the FDC network involutes, and most circulating Ag are

cleared, although FDC retain Ag trapped into long-lived immune complexes at their surface. Several groups have provided strong evidence for the continuing involvement of Ag in the maintenance of the long-lived plasma cell pool.

In addition to the CD19, CD20, CD22, and CD40 antigens expressed by most mature B cells, each B-cell subset is characterized by a set of markers and a precise histological location within B-cell follicles. In lymphoid tissue, naive (Ag-inexperienced) B cells make up the primary follicles and the mantle zone of secondary follicles. These resident naive B cells are surface Ig (SIg)M^{high}, $SIgD^{+}$, $CD44^{high}$, $CD38^{-}$, and a proportion of them also express CD23 and CD5 (Liu et al., 1994). Within GC, B cells from the dark and light zones are similarly $CD38^{high}$, $CD20^{+}$, $CD44^{low}$, $CD95^{+}$, and $bcl2^{-}$. They differ in their expression of SIg, CD10, and CD77: centroblasts are SIg^{-}, $CD10^{+}$, and $CD77^{+}$, whereas centrocytes express SIg^{+} (M, A, E, and G) but not CD10 and CD77. B cells from the dark and light zones also differ in their expression of the intracytoplasmic Ki67 antigen and surface B7 molecules: CD80 is strongly expressed in the dark zone (centroblasts, $CD86^{low}$, $Ki67^{+}$) whereas CD86 is mainly expressed in the light zone (centrocytes, $CD80^{low}$, $Ki67^{-}$) (Vyth Dreese et al., 1995). Memory B cells strongly express commutated SIg, CD44, and CD20 antigens but are $CD38^{low}$ and $CD10^{-}$, whereas plasmablasts, which express cytoplasmic Ig, are $CD38^{high}$ and $CD20^{low}$. CD62L (L-selectin) expression is also a valuable marker for recirculating naive and memory B cells. Two markers, CD27 and CD148, have been recently shown to identify memory B cells (Tangye et al., 1998) because only $CD27^{+}$ B cells seem to be able to produce commutated Ig (Agematsu et al., 1997). CD27 is also expressed by a fraction of GC B cells, which might limit its use in the characterization of lymphoid organ subsets (van Oers et al., 1993).

Cytokines are also key elements in B-cell proliferation, survival, and differentiation. Interleukin (IL)-4, and to a lesser extent IL-13, act as growth factors for BCR- or CD40-activated naive and memory B cells and rescue GC B cells by inducing Bcl-xL expression (Tuscano et al., 1996). IL-4 is locally produced by Ag-specific T cells present in the apical zone of the GC (Flynn et al., 1998). IL-4 and IL-13 decrease CD126 expression (gp80 IL-6R) but increase the production of IL-6 by activated B cells. IL-4 and IL-13 are also indispensable for the switching of naive ($SIgD^{+}SIgM^{+}$) B cells to give SIgE- and SIgG4-positive cells and for the production of IgE and IgG4 in humans. IL-10 and IL-6 support the differentiation of switched B cells of all isotypes. In combination with transforming growth factor (TGF)-β, IL-10 also drives isotype switching toward SIgG1, SIgG3, and SIgA1, the production of SIgA2 requiring direct interaction between DC and B cells (Wykes et al., 1998). Recent data suggest that IL-6 acts as a growth and survival factor for plasmablasts ($CD38^{high}$, $CD126^{high}$, $CD138^{-}$), increasing the pool of early plasmocytes ($CD38^{high}$, $CD138^{+}$). IL-6 provides a signal for survival by increasing the expression of two antiapoptotic molecules, $Bcl\text{-}_{xL}$ and Mcl1 (Puthier et al., 1999). Unlike IL-6, IL-2 is a growth factor for B lymphoblasts and increases the number of plas-

mablasts. Within GC, FDC seems to be the principal producer of IL-6, but also of the IL-8 that modulates IgE production (Kimata et al., 1995; Krzysiek et al., 1999).

CHEMOKINE/CHEMOKINE RECEPTORS CONTROL B-CELL TRAFFICKING

The mechanisms governing the organogenesis of lymphoid organs and the trafficking of B cells from the periphery to follicles and within follicles are not fully understood. Nevertheless, several chemokine/chemokine receptor pairs have been identified as key molecules controlling B-cell trafficking. The essential role of CXCR4 and its ligand, stromal cell-derived factor (SDF)-1α, in B lineage maturation in vivo has been established in sdf-1−/− and cxcr4−/− mice, which have highly impaired B lymphopoiesis and abnormally small numbers of B-lymphoid precursors in fetal liver and bone marrow (Ma et al., 1998). In the periphery, CXCR4 is expressed on most B cells and orchestrates the basal trafficking of resting B cells from the periphery to follicles. GC B cells have similar amounts of CXCR4 to naive and memory B cells, but are unresponsive to SDF-1α. After BCR triggering, the responsiveness of naive and memory B cells to SDF-1α decreases and this effect is enhanced by their concomitant stimulation by CD40 (Bleul et al., 1998). CD40 triggering alone is inefficient at regulating SDF-1α responsiveness, whereas IL-4 alone increases SDF-1α responsiveness (Jourdan et al., 1998). In the absence of CXCR5 or its ligand, BCA-1, the migration of $SIgM^+$, $SIgD^+$ naive B cells from blood into lymphoid tissue is severely impaired. Cxcr5−/− mice have no inguinal lymph nodes, few Peyer's patches, and B cells that accumulate along the sinuses of the marginal zone of the spleen rather than being organized in primary follicles (Forster et al., 1996; Voigt et al., 2000). These ectopic B cells, in close contact with FDC, nevertheless support hypermutation and antigenic selection (Voigt et al., 2000). Lymph node-like structures are induced in pancreatic islets in transgenic mice expressing BCA-1 under the control of the insulin promotor. These ectopic lymphoid structures are strictly dependent on B cells and LTα1β2 expression, confirming the essential cooperation of BCA-1 and LTα1β2 in the lymphoid neogenesis pathway (Luther et al., 2000). In normal mice and humans, CXCR5 is highly expressed on all naive and memory B cells from blood or tonsils. GC B cells also express CXCR5, but migrate poorly in response to BCA-1 (Forster et al., 1996; Kaiser et al., 1993). Short-term BCR triggering of naive B cells is sufficient to decrease CXCR5 expression. In contrast, activation of B cells by CD40 monoclonal antibodies (mAb) and IL-4 progressively decreases CXCR5 expression and responsiveness to BCA-1, neither of which are detectable after 9 days of stimulation (Bowman et al., 2000). Conflicting results have been obtained concerning the role of the CCR7/secondary lymphoid-tissue chemokine (SLC), and CCR7/EBI-1 ligand chemokine (ELC) pairs in

B-cell trafficking. B-cell follicles from lymphoid organs appear normal in *plt* mice that lack SLC and have low levels of ELC production (Vassileva et al., 1999). In ccr7−/− mice, B-cell activation and migration within lymphoid organs are abnormal. These mice spontaneously produce high levels of IgG1, IgG2a, and IgE and have enlarged GC, showing strong and inappropriate B-cell activation (Forster et al, 1999). Adoptive transfer experiments demonstrated that CCR7-deficient B cells enter into lymph nodes and Peyer's patches with a low frequency and rapidly leave the outer periarteriolar lymphoid sheath (PALS), a region essential for Ag-specific cell-cell interactions and B-cell differentiation (Forster et al., 1999). Thus, the physiological role of CCR7 seems to favor the establishment of effective T/B interactions in delaying the release of Ag-specific B cells from the PALS. ELC weakly promotes the migration of naive B cells but induces that of BCR-activated B cells (Ngo et al., 1998).

We have recently shown that naive and memory B cells, unlike GC and plasmablasts, express CCR6, the receptor of macrophage inflammatory protein (MIP)-3α (Krzysiek et al, 2000). Naive B cells express CCR6 but seem to be unable to migrate in response to MIP-3α, suggesting that CCR6 is mostly involved in control of the migration of memory B cells. BCR triggering of these B cells leads to the loss of CCR6 expression and MIP-3α responsiveness, whereas CD40 triggering or cytokine addition have no such effect. In T cells, both CCR6 expression and responsiveness to MIP-3α are restricted to the memory pool (Sallusto et al., 1999). MIP-3α is highly expressed by inflamed epithelial cells from tonsilar crypts and subepithelial regions of intestinal and lung mucosa, consistent with functional CCR6 receptors being preferentially expressed on memory cells (Tanaka et al., 1999). The esssential role of the CCR6/MIP-3α pair as a mucosa-specific regulator of humoral immunity and lymphocyte homeostasis in the intestinal mucosa has been definitively established in CCR6−/− mice (Cook et al., 2000).

Apart from fractalkine, which has both a membrane-anchored and a soluble form (Combadiere et al., 1998), chemokines control the migration of their target cells by activating a specific set of integrins and selectins. The activation of selectins results in the attachment and rolling of cells, whereas activated integrins control the firm adhesion and transendothelial migration of cells (Cyster, 1999). For example, SLC induces the firm adhesion of naive T cells to immune cell adhesion molecule (ICAM1) via β2 integrin and is a potent inducer of the α4β7-mediated adhesion of lymphocytes to Mucosal address in cell adhesion molecule (MadCAM1) expressed by high endothelial venules (HEV) (Warnock et al., 2000). The combination of a particular set of chemokine receptors and adhesion molecules therefore confers specific abilities on each cell subset to recirculate, or to interact with other cells or with the extracellular matrix. To date, little is known about the integrins and selectins used for directing B cells to specific organs and to particular areas within each organ. There is much evidence to suggest that colonization by B cells is controlled by a different combination of chemokines and TNF-related cytokines in spleen and lymph nodes (Gonzalez et al., 1998).

B CELLS IN HIV$^+$ PATIENTS

Phenotypic and functional abnormalities of B cells are detectable soon after HIV infection: most peripheral blood B cells of HIV$^+$ patients are large, cycling cells with high levels of CD38, CD70, and human leukocyte antigen (HLA)-DR antigens but no CXCR5 (Amadori and Chieco-Bianchi, 1990; Amadori et al., 1989; Forster et al., 1997; Lane et al., 1983; Martinez-Maza et al., 1987; Wolthers et al., 1996). This has led to the suggestion that these cells are blast B cells that have escaped from the GC as a result of disruption of the FDC network or failure of the apoptosis process (Forster et al., 1997). Peripheral B cells spontaneously produce large amounts of commutated Ig of various isotypes, consistent with a post-GC origin. Soon after primo-infection and at the beginning of the asymptomatic phase, 20–50% of these Ab are directed against the virus itself, but this HIV-specific part of the humoral response then progressively decreases in favor of the production of HIV-unrelated Ab and autoantibodies (Lane et al., 1983; Samuelsson et al., 1997). This suggests that the process of affinity maturation or the survival of the specific memory pool is impaired during disease progression (Amadori and Chieco-Bianchi, 1990; Bessudo et al., 1996). The physiological role of HIV-specific Ab is unclear, but several authors have suggested that high concentrations of anti-gp120 and anti-Tat antibodies slow down progression of the disease (Morris et al., 1998; Re et al., 1995; Reiss et al., 1990). Recent data from vaccination trials using synthetic Tat or recombinant gp160 proteins in experimentally infected monkeys or in HIV$^+$ patients support this idea (Cafaro et al., 1999; Caselli et al., 1999; Gallo, 1999; Gringeri et al., 1998, 1999; Polacino et al., 1999). Although, they are activated in vivo, B cells from HIV-1 patients are paradoxically poor Ig producers after in vitro challenge by recall Ag and do not significantly proliferate in response to strong B-cell mitogens or anti-IgM Ab, which trigger the BCR (Amadori and Chieco-Bianchi, 1990). In vitro, spontaneous Ig production decreases rapidly unless stimulated by the addition of IL-6. Given the essential role of IL-6 in plasmablast survival and in terminal B-cell differentiation and the high serum levels of IL-6 in HIV$^+$ patients, it was suggested that monocyte-derived IL-6 keeps the level of Ig production high in vivo (Amadori and Chieco-Bianchi, 1990). The addition of recombinant Nef and gp120 from HIV-1 to cultures of peripheral blood mononuclear cells (PBMC) was shown to increase Ig production by stimulating monocyte-derived IL-6 production (Chirmule et al., 1993, 1994). Within the lymphoid organs of HIV$^+$ patients, FDC are probably the main source of IL-6. gp120 and Tat also modulate the responsiveness of CD4$^+$ monocytes and T cells to various chemoattractants and the expression of chemokine receptors (Lafrenie et al., 1996; Secchiero et al., 1999; Wang et al., 1998), suggesting that they may affect recirculation in vivo. gp120 protein also acts as a superantigen on VH3$^+$ B cells, biasing the B-cell repertoire (Berberian et al., 1991, 1993; Karray and Zouali, 1997; Karray et al., 1998). Complex interactions occur between different regions of gp120 and VH3 and are probably independent of CD4/gp120 interactions (Neshat et al., 2000),

unlike those in T cells (Ugolini et al., 1997). Chirmule et al. have reported that the carboxy terminus part of gp41 also has B-cell stimulatory activity (Chirmule et al., 1990). Macchia et al. have shown that membrane-associated TNF-α expressed by HIV-infected T cells is also involved in maintaining B-cell activation (Macchia et al., 1993).

The sustained GC hyperplasia observed in HIV-infected patients may be partially accounted for by the continuous production of virions and viral proteins within the lymphoid tissue. Highly infectious HIV-1 virions have been shown to be present in immune complexes expressed by FDC (Armstrong and Horne, 1984; Heath et al., 1995; Joling et al., 1993; Schacker et al., 2000) and to persist, to a lower extent, under antiretroviral tritherapy (Morris et al., 1998). CD21 mAb may be useful for releasing immune complexes from FDC (Kacani et al., 2000). However, the maintenance of follicular hyperplasia during the chronic phase of the disease suggests that variations in gp120/160 proteins induce the recruitment of naive B cells directed against new antigenic epitopes. During other chronic infections, as B cells encounter the same Ag, the GC reaction is weak and renewal of the memory pool limited. Alternatively, gp120 acting, as a superantigen, and HIV-1 accessory proteins (Tat, vpr), penetrating into uninfected cells, may be involved in the abnormal B-cell activation observed in HIV^+ patients. We have recently shown that Tat impairs the proliferative response of B cells to various stimuli and their Ig production in vitro (Lefevre et al., 1999).

INFECTION OF B CELLS: IN VITRO ARTIFACT OR REALITY?

The infection of B lymphocytes by HIV-1 was first described in B-cell lines (Casareale et al., 1984; Gras and Dormont, 1991; Laurence and Astrin, 1991; Robinson et al, 1998; Rodriguez-Alfageme et al., 1998) and then in primary HIV-negative B cells subjected to in vitro infection (Gras et al., 1999; Moir et al., 1999; Poulin et al., 1994). In our hands, the HIV-1 infection of primary tonsilar B cells is complement- and virus—specific Ab-dependent (C′-ADE infection) and involves the complement receptors 2 (CD21) and 1 (CD35) and CD4 antigen (Gras et al., 1993; Legendre et al., 1996). If C3-deficient complement or normal serum is used instead of HIV^+ serum, no B-cell infection occurs. Similarly, mAb directed against CD21, CD35, or CD4 antigens and the CD4-HSA recombinant protein inhibit p24 production in B-cell cultures (Legendre et al., 1996). Although weak (<1000 molecules per cell as assessed by quantitative flow cytometry), the expression of CD4 at the surface of human B cells has also been reported by others, as well as the involvement of this antigen in the HIV-1 infection of B cells (Fritsch et al., 1998; Moir et al., 1999; Zhang and Henderson, 1994). On B cells, CD21 participates in two mutually exclusive complexes: one including CD19, CD81, and Leu 13 and the second including CD35 (Tedder et al., 1997; Tuveson et al., 1991). CD21 mAb and CD35 mAb strongly inhibit p24 production, whereas CD19 mAb does not, suggesting that

CD21/CD35 complexes may act as receptors for HIV-1, in addition to CD4. In our system of C′-ADE infection of B cells, the blockade of CD4 by four different mAb and CD4-HSA protein systematically results in partial inhibition of infection. The level of inhibition is greater in B cells with detectable CD4 (detected by flow cytometry) than in B cells with undetectable CD4. In contrast, the inhibition induced by CD21 mAb and CD35 mAb is as strong in $CD4^-$ as in $CD4^+$ B-cell populations (Legendre et al., 1996). This provides strong evidence that the CD21/CD35 complex, in addition to concentrating antibody-coated virions at the surface of FDC and B cells such that a threshold is reached allowing CD4-dependent infection, is also a true HIV receptor on B cells, mediating CD4-independent HIV entry. Triggering of the CD21/CD35 complexes may also generate intracellular signals that increase HIV-1 replication after virus entry (Matsumoto et al., 1991). Indeed, CD21 mAb and aggregated complement fragments increase B-cell activation and proliferation whereas CD35 mAb increase B-cell differentiation (Tedder et al., 1997; Weiss et al., 1987).

In our in vitro infection model, we investigated whether signals, otherwise important for B-cell differentiation within lymphoid organs, facilitated the entry of HIV-1 and favored its replication in tonsilar B cells. We observed that p24 production was greater if B cells were activated for 2 days by incubation with phorbol esters or a combination of CD40 mAb and IL-4 before infection. In contrast, levels of p24 production remained low after BCR triggering. Post-infection, p24 production is strongly increased by the combination of IL-4 and IL-2, with or without CD40 mAb (Gras et al., 1993, 1996). Similarly, Poulin et al. achieved the in vitro infection of peripheral blood B cells stimulated for 10 days in the presence of CD40L-expressing cells and IL-4, by high amounts of free HIV-1 virions (Poulin et al., 1994). In these conditions, they obtained 30–50% infected B cells, whereas we only obtained 3–10% $CD22^+$-infected B cells in our model, as shown by polymerase chain reaction (PCR) analysis of proviral sequences and membrane expression of surface gp120 (Fig. 5.1). The higher percentage of infected B cells in Poulin's study correlated with higher percentages of B cells entering the G1 phase of the cell cycle: 70–80%, versus less than 30% in our model (Banchereau et al., 1994). Using the same infection protocol, Moir et al. recently showed that the HIV-1 infection of B cells requires CD4 and CXCR4 as HIV-1 receptors and T lymphotropic or dual strains of HIV-1 (Moir et al., 1999). Although CXCR4 is expressed on all peripheral B cells, only 20–30% of CD40 plus IL-4-activated B cells are $CXCR4^+$, and only 15% of these activated B cells are also $CD4^+$. Intracellular p24 staining clearly showed that only one third of the $CD4^+$ B cells in Moir's study (i.e., 5% of total B cells) were infected with HIV-1 (Moir et al., 1999). This percentage is similar to our 1% of B cells that actively replicated HIV-1 (Gras et al., 1996) (Fig. 5.1). From these studies, it seems clear that (i) strong and sustained activation of B cells is required for in vitro HIV-1 B-cell infection; (ii) phorbol ester and CD40 triggering, which strongly stimulate nuclear factor (NF)-κB activation, favor viral replication, whereas short-term BCR trigger-

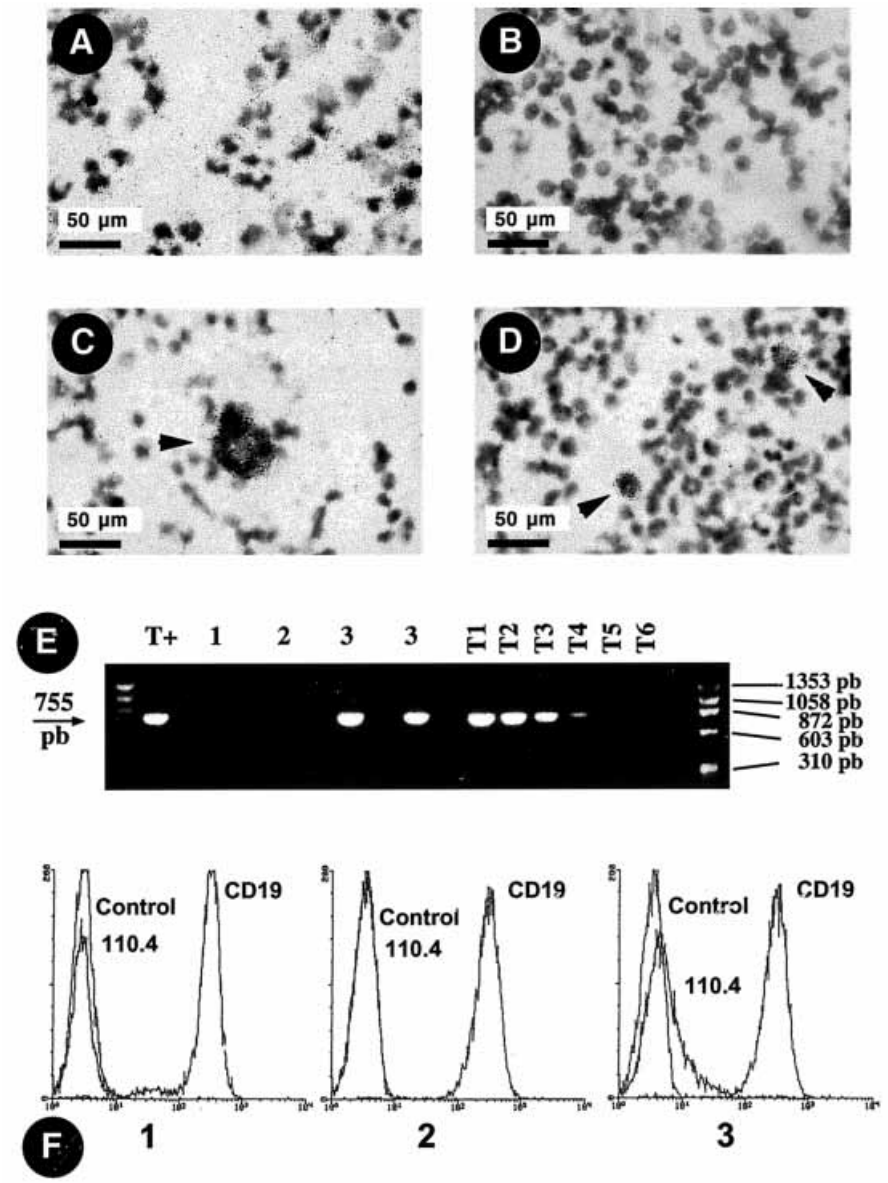

Figure 5.1. Detection of in vitro HIV-infected B cells. (**A**–**D**) In situ hybridization to detect HIV replicating in B cells. B cells were stimulated by phorbol ester for 2 days and infected, then cultured in the presence of IL-2 plus IL-4. (**A**) Chronically infected T-cell line H9V3. (**B**) Negative control, infected B cells on day 2 postinfection were hybridized with the positive strand probe. (**C**) B cells showing HIV replication on day 2 postinfection. (**D**) Isolated HIV^+ cells on day 2 postinfection. Original magnification X100. Representative of three independent experiments. (**E**) PCR detection of HIV provirus. B cells were stimulated by phorbol ester for 2 days and infected and then cultured in the presence of CD40 mAb and IL-4. One microgram of DNA was subjected to PCR. *1*, Unin-

ing, which decreases CXCR4 expression, is less effective. CXCR4-associated signaling may also be important for the maintenance of HIV replication (Cheng et al, 2000); (iii) B cells can only be infected by T-lymphotropic or dual strains of HIV-1, via CXCR4. The switch toward lymphotropic strains is also favored by high concentrations of IL-4 (Valentin et al., 1998). This suggests that B cells may be infected by HIV-1 late in the disease, accompanying the transition to the symptomatic phase and follicular involution.

In our model, the proliferation and TNFα production of infected B cells were found to increase in the presence of CD40 mAb and IL-4, with or without IL-2, whereas the highest levels of p24 production were observed in the presence IL-2 and IL-4, with or without CD40 mAb. In vivo, IL-2 production is stronger during the early phases of the disease and decreases progressively later on, whereas IL-4 production progressively increases. Thus, at all stages of the disease, HIV-1 replication may be sustained by these cytokines. IL-10 decreases p24 production induced by most of these stimuli by 34–72%, but only inhibits IL-4-mediated and IL-2-mediated cell proliferation (Gras et al., 1996). IL-10-mediated inhibition of HIV-1 replication has also been reported in macrophages, in which it seems to counteract NF-κB activation (Wang et al., 1995). IL-10 is produced during the late phase of the disease and may therefore antagonize IL-2- or IL-4-mediated HIV replication without decreasing B-cell proliferation or Ig production. Thus, our results show that cell proliferation, cytokine production, and HIV-1 replication in B cells are under the control of different pathways. In contrast to Poulin et al., who showed a significantly lower level of proliferation for their HIV-infected B cells (Poulin et al., 1994), we found the levels of proliferation of HIV-infected and mock-infected cells to be similar. Nevertheless, it is possible that the decrease in cell proliferation was undetectable due to the low percentage of infected B cells we obtained. We also observed no syncytia formation in our model, whereas Moir et al. detected syncytia but only after isolation of the $CD4^+$ fraction (Moir et al., 1999). In vivo, syncytium formation and decrease in cell proliferation might result in the rapid death of HIV-1-infected B cells, contributing to follicle involution observed in HIV^+ patients.

Although B cells are susceptible to HIV-1 infection in vitro and support active HIV-1 replication, the in vivo infection of B cells has not yet been demonstrated. This is intriguing given the strong expression of CXCR4 on most B cells and the presence of CD4 on at least a proportion of B cells. The best

◄

fected B cells; *2*, mock-infected B cells, day 4 postinfection; *3*, c′-ADE-infected B cells, day 4 postinfection; *T1–6*, 10-fold dilutions of 8^E5-LAV cell DNA in PBMC DNA. T1 contains 17,300 proviral copies in 1 μg of DNA. Representative of three independent experiments. (**F**) Flow cytometric analysis of HIV-1 gp120 envelope protein expression on c′-ADE-infected B cells. Phorbol ester-activated B cells were submitted to HIV infection in c′-ADE conditons and cultured for 2 days in the presence of cytokines. Indirect immunofluorescence of gp120 was performed with 110.4 mAbs. B cells were stained with CD19 mAb and mouse IgG as positive and negative control, respectively. *1*, Infected B cells, 1 h postinfection; *2*, uninfected B cells, day 2 postinfection; *3*, infected B cells, day 2 postinfection. Representative of two experiments.

candidates for infection by HIV-1 in vivo are activated memory B cells and centrocytes. These cells produce commutated Ig and are located in the vicinity of T cells responsible for strong CD40 triggering and producing cytokines (IL-2, IL-4, IL-10). However, CXCR4 is not fully functional on GC B cells and stimulation of their BCR by FDC-trapped HIV-1 complexes would further decrease CXCR4 expression. In the GC microenvironment, B-cell infection is more likely to be due to these highly infectious opsonized virions presented by FDC, possibly through the CD21/CD35 complex. We have not investigated the requirement for chemokine receptors for C′-ADE HIV infection. Alternatively, it is possible that mature B cells of any subset, once suitably activated, may be infected by HIV-1. We have indeed shown that although phorbol ester-activated B cells express less CD4 and complement receptors than resting B cells, they are more susceptible to HIV-1 infection.

THE CD86/CD28 PAIR CONTROLS HIV-1 REPLICATION IN INFECTED T CELLS

At each step of the T-dependent humoral response, a reciprocal dialogue takes place between Ag-triggered B and T cells within the lymphoid tissue (Kelsoe, 1996). The small pool of infected $CD4^+$ T cells that produce HIV-1 RNA are located in and around hyperplastic GCs of secondary follicles, in close contact with Ag-presenting cells (APC), including DC in follicular and extrafollicular areas and activated B cells within GC (Biberfeld et al., 1986; Fox et al., 1991; Hufert et al., 1997). In blood and lymphoid tissue, interactions between DC and $CD4^+$ T cells allow de novo infection of T cells and increase viral replication in T cells (Pinchuk et al., 1994; Pope et al., 1995; Weissman et al., 1996). Activated B cells, particularly GC B cells, also provide costimulatory signals that support HIV-1 replication and T cell activation in in vitro HIV-1-infected T cells (Krzysiek et al., 1998). At the optimal ratio (i.e., 10 B cells per T cell), activated B cells were found to increase HIV-1 replication strongly, when p24 production was assessed or by using reverse transcriptase (RT) activity as a marker. On day 8 postinfection, RT activity was 25 to 31 times higher with B cells than without B cells. Similarly, more than 94% of $CD4^+$ T cells expressed gp120 at their surface when cultured in the presence of B cells, whereas only 61% of $CD4^+$ T cells were $gp120^+$ in their absence (Fig. 5.2).

In addition to T-cell receptor (TCR) stimulation, costimulation via CD28 triggering is required for a full T-cell response (Boise, et al, 1995; Boussiotis et al, 1996; June et al 1994). CD28, expressed on most $CD4^+$ and half of all $CD8^+$ T cells, interacts with two ligands present on most APC, the CD86 and CD80 molecules (Boussiotis et al., 1996). Although CD80 and CD86 have the same natural counterreceptors, CD28 and CTLA-4, they seem to control differently the TH2/TH1-type T-cell response (Freeman et al., 1995; Kuchroo et al., 1995), CTL generation, autoimmunity, and tumor growth (Boussiotis et al., 1996). These two B7 molecules also exhibit different patterns of B-cell surface

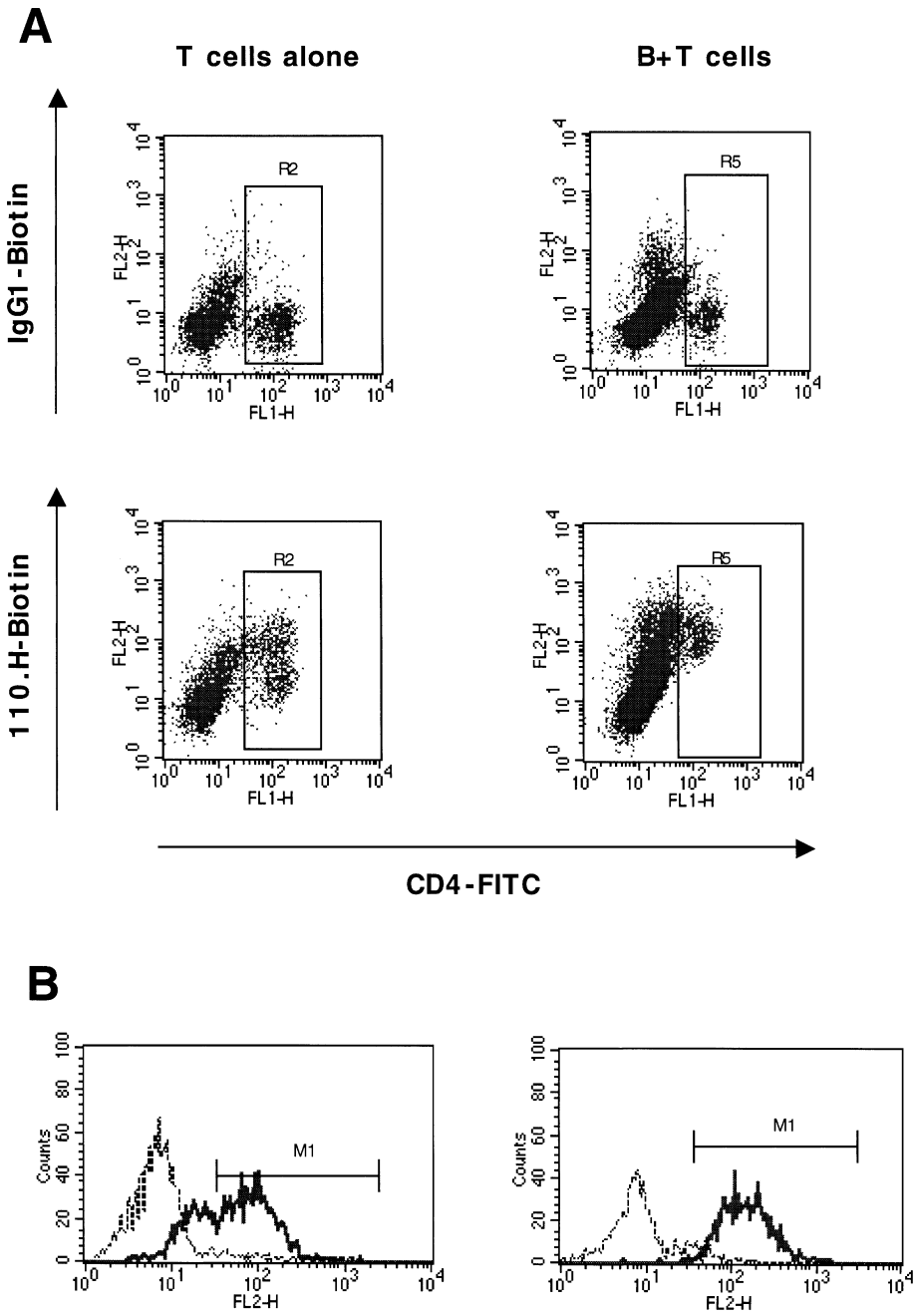

Figure 5.2. B/T cocultures increase the percentage of gp120^{+}CD4^{+} T cells. (**A**) 10^4 HIV-1 infected T cells were cultured alone or with 10^5 phorbol dibutyrate-activated B cells for 4 days. On day 4, cell populations were analyzed for cell surface gp120 expression. Two-color FACScan analysis shows staining with IgG1-biotin and CD4-FITC (fluorescein isothiocyanate) (*upper plots*) and 110.H-biotin and CD4-FITC (*lower plots*). (**B**) Expression of gp120 (*bold line*) and IgG1 (*dotted line*) in gated CD4^{+} T cells (R2 or R5 gates) is shown. Representative of two independent experiments.

expression: CD86 is expressed earlier than CD80 after B-cell activation and is mostly detected on centrocytes, whereas CD80 is most strongly expressed on centroblasts (Vyth Dreese et al., 1995). CD80 and CD86 have been detected on T cells from HIV-infected patients (Wolthers et al., 1996) and patients with autoimmune disorders (Wyss-Coray et al., 1993). Their high level of expression on T cells, probably due to continuous immune activation, is generally associated with low levels of CD28 expression (Haffar et al., 1995). Nevertheless, CD86 expression seems to be restricted to the $CD28^+$ cells. Despite its low level of expression on HIV-infected T cells, the stimulation of CD28 greatly increases HIV-1 replication and T-cell growth (Asjo et al., 1993). Consistent with recent data suggesting that CD80 and CD86 have nonoverlapping physiological functions (Freeman et al., 1995), we showed that HIV-1 replication induced by T/B costimulation was mainly controlled by CD86-mediated interactions. Indeed, CD86 mAb inhibited HIV-1 replication by 80% whereas CD80 mAb gave only 30% inhibition. Similarly, B-cell-induced T-cell proliferation was inhibited by 50% in the presence of CD86 mAb but by $<10\%$ with CD80 mAb. Additive effects were observed in the presence of CD86 mAb and CD80 mAb, with HIV-1 replication inhibited by $\geq 90\%$ and T-cell proliferation inhibited by 50–70%. The effects of these mAb on HIV-1 replication reflect their capacities to elicit distinct profiles of cytokines. CD86 mAb and CD80 mAb inhibited IL-2 production by 77–98%. TNF-α production was inhibited by 84% and 43% in the presence of CD86 mAb and CD80 mAb, respectively, whereas interferon (IFN)-γ production was inhibited by 54% by CD86 mAb but was unaffected by CD80 mAb. This suggests that, in the absence of IL-2, the residual TNF-α and IFN-γ present in CD80 mAb-treated cultures were sufficient to maintain a significant level of HIV-1 replication and T-cell proliferation. This hypothesis is supported by the fact that the addition of neutralizing anti-TNF-α mAb increased CD80-induced inhibition whereas recombinant TNF-α partially reversed the CD86-induced inhibition of p24 production. No significant difference in intracellular signaling has been demonstrated between CD80- and CD86-mediated triggering of counterreceptors (Rudd, 1996), but it is well known that these molecules cause different functional outcomes in T cells (Boussiotis et al., 1996; Kuchroo et al., 1995). Although both HIV-1 replication and T-cell proliferation were increased by B-cell costimulation, the rate of viral replication did not closely correlate with the level of T-cell proliferation. Even in the presence of both CD80 mAb and CD86 mAb, which inhibited almost all IL-2 and TNF-α production and two thirds of IFN-γ production, T-cell proliferation only decreased by 60%. Thus, intrinsic costimulatory signals delivered by CD28/B7 interactions may independently control virus replication and T-cell proliferation. Independent control of cell proliferation and viral replication by cytokines has also been suggested by Smithgall et al. (1996). By increasing T-cell proliferation, CD86-dependent interactions may not only enhance HIV-1 production in already infected T cells but may also favor de novo T-cell infection. These results suggest that CD86-mediated interactions may be essential for B cell-driven active in situ replication of HIV-1 in GC T cells. The largest

HIV-1 reservoir and viral replication site colocalizes with the area of highest CD86 expression, within the light zones of GC. Thus, CD86-mediated interactions, which take place during the response to each T-dependent antigen, also stimulate HIV-1 replication in T cells.

IN SITU EXPRESSION OF CD80 AND CD86 WITHIN GC OF HIV+ PATIENTS

As CD80- and CD86-mediated interactions differentially regulate HIV-1 replication in in vitro infected T cells, we compared the in situ expression of CD80 and CD86 in HIV^+ and HIV^- lymphoid organs. We observed enlarged, irregularly shaped GCs surrounded by a limited, frequently disrupted, IgD^+ mantle zone in hyperplastic HIV^+ lymph nodes (LNs), as previously shown (Baroni and Uccini, 1990; Racz et al., 1986) (Fig. 5.3*F*). $CD4^+$ T cells were scattered in the GC of HIV^+ patients rather than being limited to the light zone as in normal tonsils and HIV^- LNs. There were significantly more $CD57^+$ cells in the extrafollicular zone and in $CD8^+$ T cells in the GC of hyperplastic HIV^+ LNs, consistent with previous data (Borthwick et al., 1994; Koopman et al., 1997) suggesting that these infiltrated $CD8^+$ T cells are involved in FDC network disruption and the destruction of HIV-infected cells (Koopman et al, 1997). $CD8^+$ cells also produce chemokines that suppress HIV infection (Cocchi et al., 1995) and regulate T-cell homing, so chemokines/chemokine receptors may also impair B-cell homing (Forster et al., 1997) or function (Kimata et al., 1996). The increase in CD38 expression in the extrafollicular zone in HIV^+ patients is consistent with the strong and persistent activation of T cells during HIV infection (Kestens et al., 1994). We also found high levels of CD95 expression in the extrafollicular zone of HIV^+ LNs. This probably increases T-cell sensitivity to apoptosis, as reported for peripheral blood T cells (Silvestris et al., 1996).

The FDC network, uniformly distributed in normal and hyperplastic HIV^- GC, was frequently disrupted in HIV^+ GC. Similar results were obtained by Baroni et al. (Baroni and Uccini, 1990). There was no significant change in the pattern of expression of most B-cell markers, but polarization of the HIV^+ GC was lost, as shown by Ki67, CD10, and CD77 staining, showing a random distribution of centroblasts throughout the GC (Fig. 5.3*E*). There is no obvious marker for centrocytes but they do not express Ki67 and CD77, are $CD38^+$ and $CD95^+$, and have high levels of CD86. Based on this pattern, we observed that centrocytes, present only in the light zone of normal and hyperplastic HIV^- GC, were distributed throughout the GC of HIV^+ patients (Fig. 5.3*F*). It is therefore unlikely that a particular population of GC B cells increases during HIV-induced hyperplasia.

$CD86^+$ cells limited to the light zone of normal tonsils and HIV^- hyperplastic LNs were dispersed throughout the GC in HIV^+ patients (Fig. 5.4*F*) but the intensity of CD86 staining was similar for all sections studied. Unlike

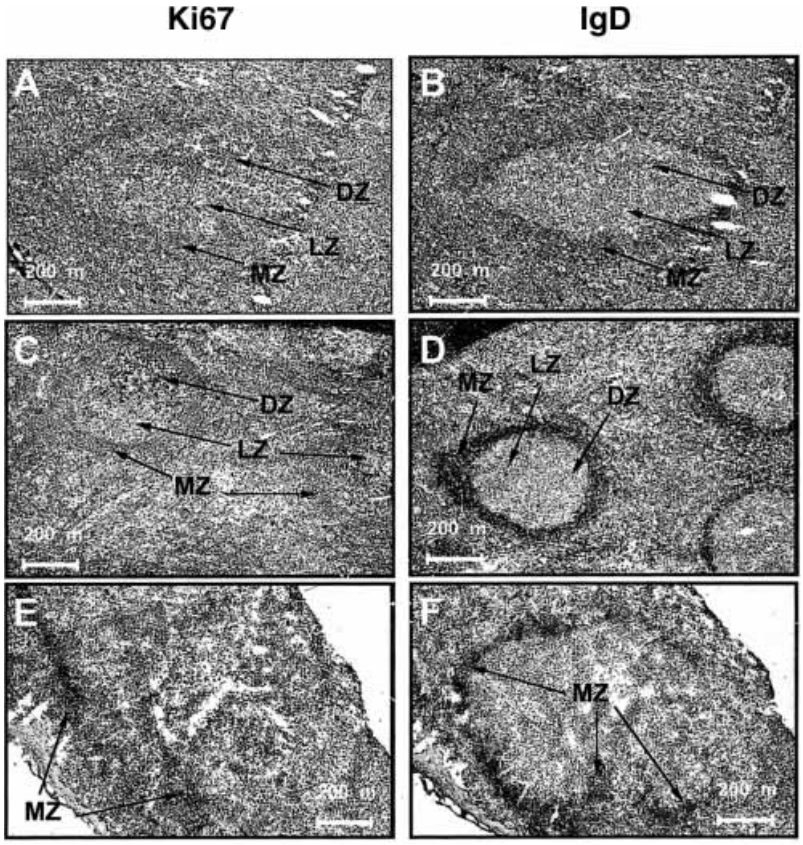

Figure 5.3. Loss of polarization in hyperplastic HIV$^+$ germinal centers. Ki67 and IgD labeling of normal tonsil (**A, B**), hyperplastic HIV$^-$ (**C, D**), and HIV$^+$ (**E, F**) lymph node sections. Labeling was detected with 3,3′-diaminobenzidine chromogen. Sections were counterstained with Mayer's hemalun and mounted. Original magnification (A–F): X25. *LZ*, Light zone; *DZ*, dark zone; *MZ*, mantle zone.

Vyth-Dreese et al., who showed that CD80 expression was restricted to the dark zone of GC (Vyth Dreese et al., 1995), we found that CD80 staining overlapped dark and light zones in tonsil sections (Fig. 5.4*A*). This may be due to the use of different CD80 mAbs, BB1 or L307.4 versus B7.24 mAb. CD80 staining was weak or absent in all HIV$^+$ LNs but was observed in all HIV$^-$ hyperplastic GC. We detected CD86 and CD80 on extrafollicular DC from most sections. Cell suspensions from normal tonsils and from the LNs of three HIV$^+$ patients were double-labeled with CD19 mAb and CD80 mAb or CD86 mAb. CD80 and CD86 expression was similar on CD19$^+$ B cells from tonsils whereas CD80 expression was lost or decreased on the surface of CD19$^+$ B cells from the three HIV$^+$ patients tested (Fig. 5.5). In contrast, CD86 was similarly expressed in these patients and in controls. As DC and FDC were scarce in cell

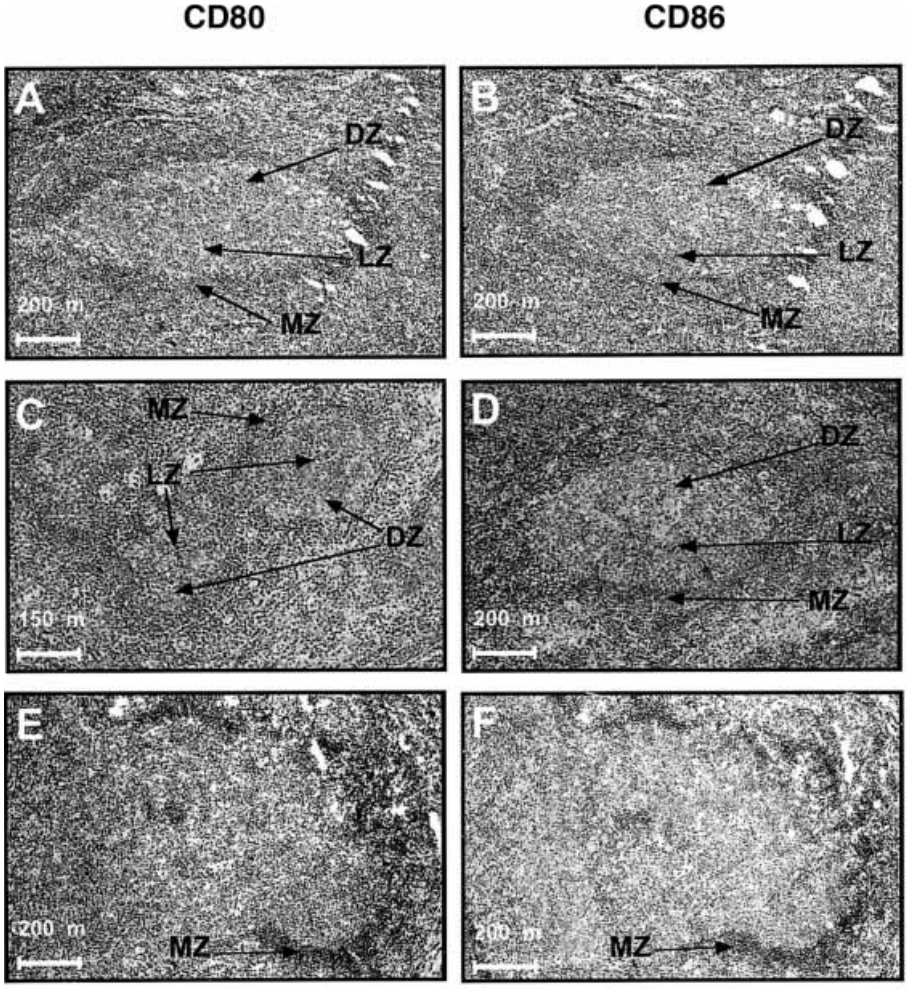

Figure 5.4. Low level of CD80 in hyperplastic HIV+ lymph nodes. CD80 and CD86 labelings of normal tonsil (**A, B**), hyperplastic HIV− (**C, D**), and HIV+ (**E, F**) lymph node sections. Original magnification (A–F): X25. *LZ*, Light zone; *DZ*, dark zone; *MZ*, mantle zone.

suspensions, it was impossible to determine whether CD80 and CD86 expression was changed on these cells. There was no correlation between the magnitude of the decrease in CD80, the stage of the disease, antiretroviral therapy, and the number of circulating $CD4^+$ T cells. Previous studies have reported that peripheral HIV^+ T cells express CD80 and CD86 antigens (Wolthers et al., 1996). In contrast, we did not detect these Ag on T cells from HIV^+ LN sections.

Studies in vitro have shown that CD80 is a late activation marker of APC requiring CD86/CD28 and CD40/CD40L(CD154) interactions for its expres-

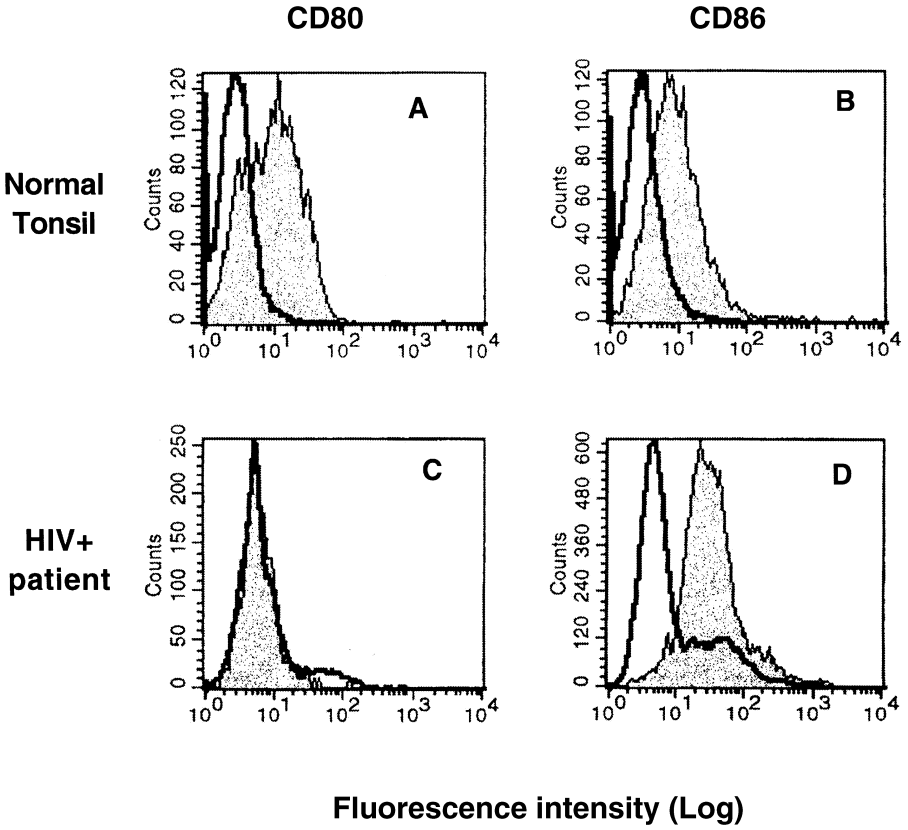

Figure 5.5. CD80 expression on $CD19^+$ B cells. Cell suspensions from one tonsil and one HIV^+ patient were labeled with phycoerythrin (PE)-CD80 and fluorescein isothiocyanate (FITC)-CD19 mAbs (**A and C**) or with FITC-CD86 and PE-CD19 mAbs (**B and D**). Analysis was performed on 10,000 viable cells. Unshaded and shaded histograms are the control and CD80 or CD86 labeling, respectively.

sion (Boussiotis et al., 1996; Somoza and Lanier, 1995). Thus, the low level of CD80 in HIV^+ patients might result from inadequate T/B or FDC/B interactions and/or being associated with GC depolarization. Soluble factors released by T cells or HIV-presenting FDC may also affect CD80 expression on B cells (Reiser and Stadecker, 1996; Somoza and Lanier, 1995). The loss of CD80 may lead to more CD86/CD28 interactions, increasing HIV-1 replication within the GC (Krzysiek et al., 1998), and leading to a shift toward T-helper 2 (TH2) T-cell responses, as previously reported (Klein et al., 1997). It has been suggested that preferential interactions occur between CD80 and CTLA-4 antigens (Boussiotis et al., 1996). CTLA-4 may exert feedback control on the T-cell response (Tivol et al., 1995), so the loss of CD80 may be involved in maintaining hyperplasia. Thus, hyperplasia affects both centroblast and cen-

trocyte populations in HIV^+ LNs. Despite the strong GC depolarization typical of HIV^+ hyperplasia, the expression of most B-cell markers is similar in HIV^+ and HIV^- lymph nodes. The most striking result is the loss of CD80 from the GC, with CD86 levels unchanged. Experiments are underway in experimentally infected macaques to clarify whether (i) GC depolarization and the loss of CD80 expression in GC occur progressively or are already detectable after primo-infection; (ii) the development of these B cell abnormalities is dependent on T-dependent Ag stimulation; and (iii) antiretroviral tritherapy restores expression of CD80 in lymphoid tissue. Preliminary results in simian-human immunodeficiency (SHIV)-infected macaques suggest that the development of GC hyperplasia requires repetitive Ag challenge in addition to viral infection.

HIV-1 TAT PROTEIN: A POTENT MODULATOR OF B-CELL FUNCTIONS

Previous reports have shown that soluble gp120, Vpr, Nef, and Tat are present as biologically active extracellular proteins released by infected cells in HIV^+ patients (Westendorp et al., 1995). Extracellular Nef and gp120/160 increase terminal differentiation of B cells into Ig-producing cells by increasing T/B interactions and monocyte-derived IL-6 production (Chirmule et al., 1993, 1994). Extracellular Tat, readily taken up by uninfected cells and targeted to the nucleus (Frankel and Pabo, 1988), stimulates the growth of Kaposi's sarcoma cells, potentiates the anergy and apoptosis of uninfected T cells, and promotes chemotaxis and invasive behavior by monocytes (Albini et al., 1998; Lafrenie et al., 1996; Rubartelli et al., 1998). In B-cell lines, Tat also modulates the production of cytokines and their receptors (Puri and Aggarwal, 1992; Sharma et al., 1995). It also increases CD95 expression on peripheral B cells during short-term cocultures with T cells and monocytes (Huang et al., 1997). Recent data also showed that viral protein R (Vpr) acts as a proapoptotic agent in monocytes and T cells (Jacotot et al., 2000). Thus, these accessory proteins, locally produced in the lymphoid organs of HIV^+ patients, might directly act on primary B cells and contribute to the B-cell abnormalities observed in vivo.

We have recently established that extracellular Tat, unlike recombinant Nef, exerts a direct effect on all primary B-cell subsets, but differentially modulates the anti-IgM Ab- and CD40 mAb-induced proliferation of naive, memory, and GC B-cell subsets isolated from the lymphoid organs of HIV^- donors (Fig. 5.6) (Lefevre et al., 1999). Indeed, Tat strongly inhibits the proliferation of BCR-triggered naive and memory B cells but has no effect on their CD40 mAb and cytokine (IL-4 or IL-2 plus IL-10) mediated cell proliferation. In contrast, Tat doubles the GC B-cell proliferation induced by CD40 mAb and IL-4. These functional effects of Tat are (i) dose-dependent with a maximal effect obtained between 0.5 and 1 μg/ml (0.05–0.1 μM) for 10^6 cells/ml (these concentrations are higher than the serum concentration, 0.01–0.5 nM, reported for $HIV\text{-}1^+$ patients but similar to those used in vitro with T cells, 0.5 nM to 2 μM; (ii)

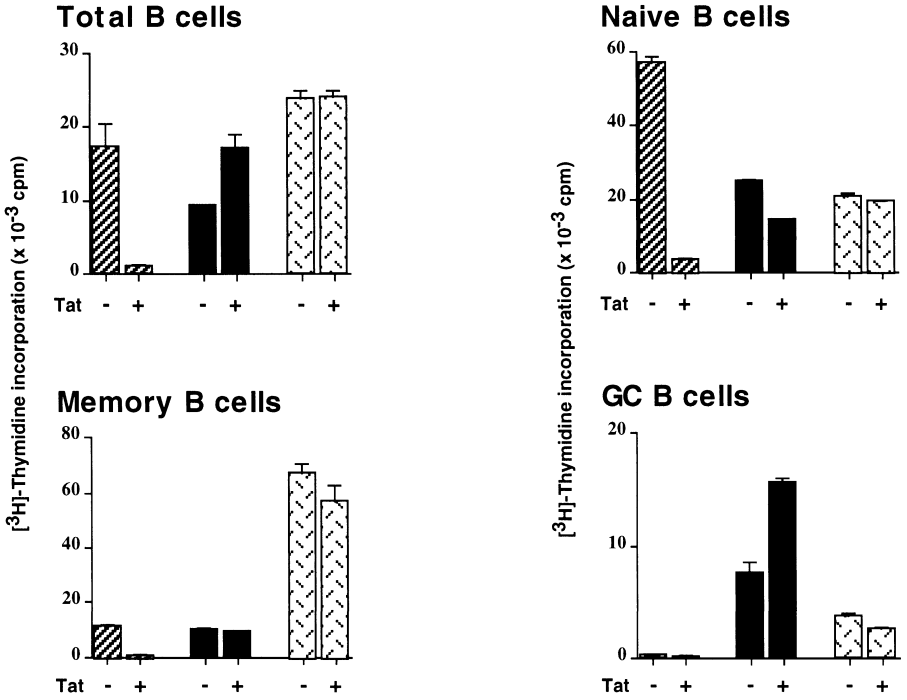

Figure 5.6. Tat differentially modulates cell proliferation of various B-cell subsets. Total, naive memory and GC B cells were stimulated by anti-IgM Ab and IL-4 (*medium shading*), CD40 mAb and IL-4 (*dark shading*), or CD40 mAb and IL-2 plus IL-10 (*light shading*) in the presence or absence of 0.5 μg/mL wild-type Tat. Results of [^{3}H]-thymidine incorporation are expressed as mean counts per minute (*cpm*) ± SD of triplicates determinations. Values are representative of five independent experiments.

time-dependent because the maximal effect of Tat required the presence of this molecule during the first hours of the B-cell stimulation; (iii) blocked by the prior incubation of Tat with heparin, with an ID_{50} (1 μg/ml heparin) similar to that reported for Tat-induced HIV-1 transactivation (Rusnati et al., 1997); (iv) similar for wild-type recombinant and synthetic Tat proteins from various strains (IIIb, BH10, Mal, Eli) and for two mutated synthetic Tat devoid of transactivating activity (Tat Oyi and CmC Tat Bru) (Fig. 5.7) (Lefevre et al., 1999). This shows that the cysteine-rich region of Tat, essential for transactivation of the HIV-1 LTR, does not control the effects of Tat on B cells.

The Tat-induced inhibition of naive and memory B-cell proliferation is not due to apoptosis but to the arrest of IgM-mediated B-cell activation before the G1 to S phase transition. Indeed, Tat modulates [^{3}H]-thymidine and [^{3}H]-uridine incorporation to the same extent. The addition of Tat does not change the percentage of $CD69^+$- and $CD71^+$-activated B cells but increases that of $CD95^+$ B cells (Fig. 5.8). Tat decreases the amounts of cyclin D3, cyclin E, cdk4, and cdk6 in IgM-activated B cells but not in CD40-activated B cells

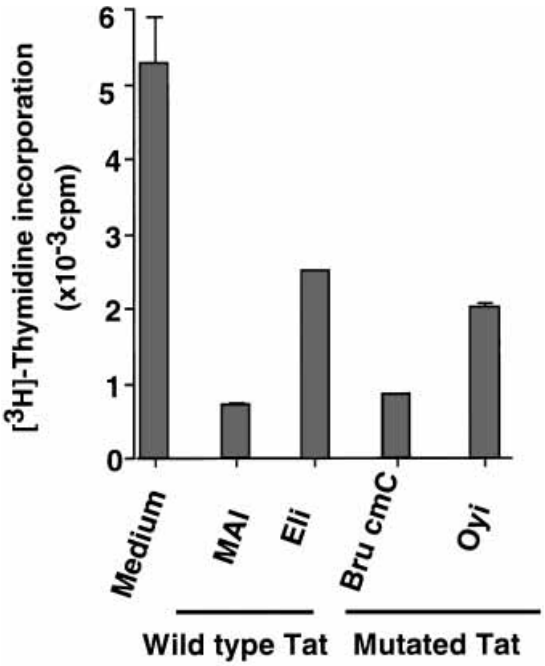

Figure 5.7. Mutated Tat proteins devoid of transactivating activity are as efficient as wild-type Tat proteins at inhibiting anti-IgM-mediated B-cell proliferation. Naive B cells were stimulated for 3 days by anti-IgM Ab and IL-4 in the presence of 1 μg/mL synthetic wild-type Tat proteins (Tat MAL, Tat Eli) or synthetic Tat proteins mutated in their cystein–rich region (Tat Bru cmC, Tat Oyi). Results of [^{3}H]-thymidine incorporation are expressed in mean counts per minute (*cpm*) ± SD of triplicates determinations. Data are representative of five independent experiments.

(E. A. Lefevre, personal communication). Kundu et al. showed that Tat prolongs the G1 cell cycle by decreasing the kinase activity of cdk2/cyclin E complexes in glial cells (Kundu et al., 1998), but the mechanism by which Tat impairs B-cell progression is unknown. As Tat regulation of the proliferation of naive/memory B cells differs according to the stimulus used for their activation, it may interact with specific cytoplasmic mediators or nuclear factors. Even if added extracellularly, Tat is rapidly translocated into the nucleus, suggesting that it is most likely to interact with nuclear mediators, such as NF-κB (Rubartelli et al., 1998). The higher level of CD95 expression on anti-IgM-activated naive and memory cells after treatment by Tat may favor their apoptosis through CD95/CD95L interactions. In other respects, stimulation by CD40 mAb and IL-4 is a potent survival signal for GC B cells that leads to an increase in the expression of Bcl-$_{xL}$ (Tuscano et al., 1996). It is thus possible that Tat acts on GC B cells, in up-regulating the expression of these antiapoptotic molecules. Alternatively, Tat may increase the expression of IL-4 receptors on GC B cells, thereby increasing their sensitivity to IL-4 (Puri and Aggarwal, 1992). Whereas CD40 mAb and IL-2 plus IL-10 induce strong B-cell prolifer-

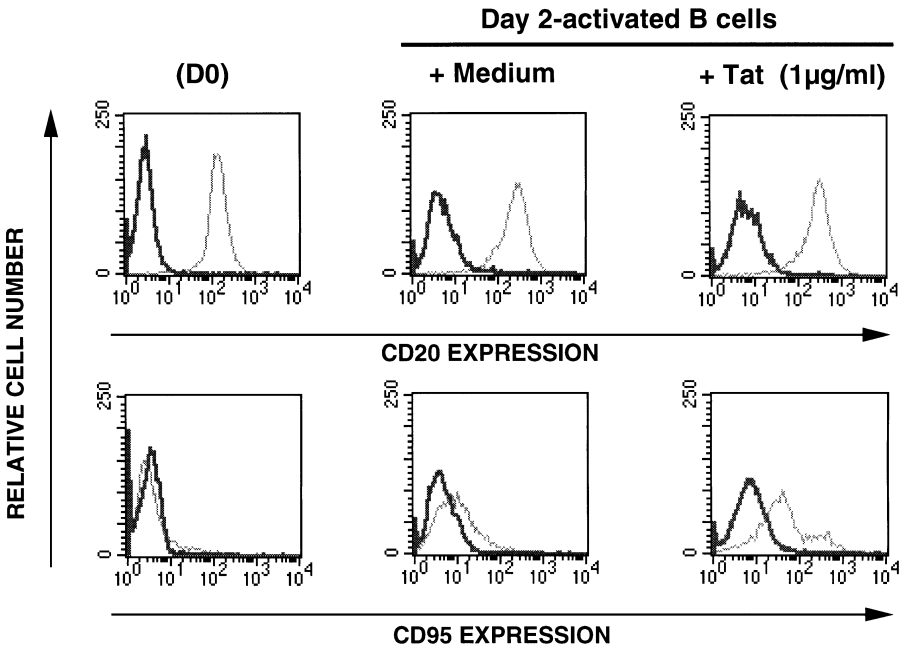

Figure 5.8. Extracellular Tat increases CD95 expression on BCR-triggered naive B cells. Naive (IgD^+) B cells were stimulated for 2 days by anti-IgM Ab and IL-4 in the presence or absence of wild-type Tat (1 µg/mL). Unstimulated (D0) and day 2-activated B cells were stained with CD20-FITC (fluorescein isothiocyanate) or CD95-PE (phycoerythrin) mAb. Data are representative of three independent experiments.

ation and Ig production, only Ig production is inhibited by Tat. Tat decreases IgA, IgG, and IgM production by 40–60% without impairing CD40-induced isotype switching. This suggests that Tat decreases the survival or terminal differentiation of plasma cells.

As Tat exerts some of its biological activity by interfering with cytokine production (Rubartelli et al., 1998), we assessed its effect on the production of several B-cell-derived cytokines. No significant change in IL-8, IL-10, and TGF-β production was observed after Tat addition in our experimental conditions. The addition of Tat slightly affected the production of MIP-1α, MIP-1β, TNF-α, and IL-6 in CD40 mAb and IL-4-stimulated cultures but strongly decreased their production in anti-IgM Ab-stimulated cultures. It seems unlikely that Tat controls B-cell proliferation via the modulation of cytokines because: (i) Tat increases CD40 mAb and IL-4-induced cell proliferation but not cytokine production and (ii) addition of recombinant MIP-1α, MIP-1β, TNF-α, or IL-6 does not abolish the Tat-induced inhibition of anti-IgM proliferation. However, the low level of MIP-1α and MIP-1β production may impair the recruitment of $CD4^+CD45RO^+$ helper T cells and interfere with T/B interactions, leading to abnormal T-cell-dependent maturation of the B-cell response

(Krzysiek et al., 1999). It is also unknown whether Tat modulates the responsiveness of B cells to SDF-1α, BCA-1, ELC, SLC, fractalkine, or TECK (thymus-expressed chemokine), or the expression of their respective receptors, thereby contributing to the abnormal relocation of B cells observed in HIV^+ patients.

Tat, by increasing GC B-cell proliferation, may play an important role in HIV-associated centrofollicular hyperplasia. This hypothesis is supported by the data of Kundu et al. showing that *tat* transgenic mice develop centrofollicular hyperplasia and B lymphomas (Kundu et al., 1999). Tat may also be involved in the loss of the mantle zone in follicles and the progressive decrease in the B-cell repertoire, by inhibiting cell cycle progression, Ag-specific T/B interactions, and cytokine production in naive B and memory cells. Our data raise numerous questions concerning the use of Tat as a therapeutic or vaccination agent in HIV^+ patients. Several groups have reported that vaccination with Tat proteins that have mutations in the cysteine-rich region, and are thus incapable of transactivation, induces the production of specific Ab and limits the progression of the disease (Cafaro et al., 1999; Caselli et al., 1999; Gallo, 1999; Gringeri et al., 1998, 1999). However, a recent article reported that similar mutated, but not wild-type, Tat proteins down-regulate the expression of HLA Class I in T cells in vivo (Tosi et al., 2000). We have also found that these mutated Tat proteins are as efficient as wild-type Tat at impairing the B-cell response (Lefevre et al., 1999). Based on these data, we think that repetitive Ag challenges with T-dependent Ag should be included in all future therapeutic trials using Tat and that the B-cell compartment in the lymphoid organs of these monkeys should be carefully studied. From now on, it would be most useful to design Tat proteins not only devoid of transactivating activity but also unable to enter B cells (RGD or basic regions) or to be targeted to the nucleus (nuclear localization signal region). Knowledge of the mechanisms by which Tat exerts its effects in B cells would provide new targets for therapeutic trials. An alternative possibility for vaccination is the use of cyclic peptides derived from Tat, as suggested by Friedler et al. (Friedler et al., 2000).

HIV-1 AND B-CELL LYMPHOMAGENESIS

In the absence of antiretroviral therapy, HIV^+ patients frequently develop high-grade non-Hodgkin's lymphoma, known as AIDS-associated lymphoma. Most of these lymphomas are of B-cell origin and there is frequent involvement of extranodal sites, 25% in the central nervous system (CNS) and 75% in the periphery (Ng and McGrath, 1998), giving rise to two histological groups: large-cell and immunoblastic lymphoma and Burkitt's lymphoma. Studies of Epstein-Barr virus (EBV) expression and of Ig and c-myc rearrangements have shown that large-cell lymphomas are predominantly polyclonal and usually lack EBV and c-myc rerrangements and Burkitt's lymphomas are monoclonal with a high frequency of rearranged c-myc genes, whereas CNS lymphomas

consist of uniformly monoclonal B cells, expressing EBV but not rearranged c-myc genes (Ng and McGrath, 1998). Patients with large-cell lymphoma survive longer and the length of survival correlates with CD4 T-cell count, whereas patients with monoclonal EBV^+ lymphomas have a very poor prognosis. The interactions between HIV, EBV, and *c-myc* that lead to malignant transformation of B cells in vitro are known, but the presence of HIV within tumor cells in vivo is poorly documented and remains controversial. Indeed, to our knowledge, only two articles have suggested that HIV-1 is present in lymphomatous B cells or in B cells from hyperplastic lymph nodes (Astrin et al., 1992; Prévot et al., 1993). Most AIDS-related lymphomas are HIV^-, suggesting that lymphomagenesis does not require B-cell infection by HIV-1, although viral proteins probably increase the selection pressure on B cells.

Data on AIDS-related lymphoma Igs, VH use, and somatic mutations provide evidence that these cells may be outgrowths of polyreactive B cells that escape normal B-cell selection (Bessudo et al., 1996; Ng et al., 1994, 1997; Przybylski et al., 1996). These B cells, destined to become malignant in HIV-infected patients, are probably present early in the disease, during the hyperplastic stage. The loss of B-cell differentiation control facilitates their survival and expansion in the host. Chronic stimulation is likely to result in polyclonal lymphomas that may then evolve into monoclonal processes. The studies on AIDS-related lymphoma and the progressive loss of specific B-cell memory observed in HIV^+ patients suggest that most promalignant B-cell clones arise from the normal hypermutation process but that the impairment of positive or negative selection allows them to survive. Tat may favor the survival of these cells by increasing the expression of $Bcl\text{-}_{xL}$ or of other antiapoptotic molecules, whereas the loss of GC organization may decrease the frequency of CD95/CD95L interactions. It should be noted that *tat*-transgenic mice develop centrofollicular lymphomas (Kundu et al., 1998) even in this absence of HIV-1 infection.

CONCLUSION

Although the in vivo infection of B cells by HIV-1 remains to be demonstrated, it is clear that B cells are direct targets of viral HIV-1 proteins. Thus, in addition to promoting the T-cell response and $CD4^+$ T survival in HIV patients, it seems to be important in protecting the integrity of the humoral response and B-cell functions. Monkeys are useful for studying the B-cell compartment in blood and within the lymphoid organs during HIV infection, in the presence or absence of Ag stimulation. Alternatively, therapeutic trials in experimentally infected monkeys could evaluate B-cell functions in general, and the affinity of Ab raised against viral proteins in particular.

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6

Cytotoxic T-Cell (CTL) Function in HIV Infection

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INTRODUCTION

Adaptive immune responses to infectious agents have traditionally been classified into humoral and cellular immune responses. Humoral immunity, mediated by B cells is directed at extracellular pathogens whereas cellular immunity, mediated by T cells is largely directed at intracellular pathogens including most viruses. There is considerable overlap as these two types of immune responses are not independent of each other. T cells produce cytokines that activate B cells playing a role in humoral immunity. B cells in turn influence cellular immunity through antibody dependent cytotoxicity (ADCC) and function as highly efficient antigen presenting cells, especially for proteins that bind to their surface immunoglobulins (Abdullah and Greenman, 1999; Patil and Gupte, 1995).

Cellular immunity is largely a function of lymphocytes and involves multiple mechanisms and cell types. The ultimate goal of this immune response is to remove the invading pathogen. The most well-defined mechanisms involved include secretion of cytokines such as interferons (IFN) and interleukins (IL) as well as killing via the granule-exocytosis and the Fas/Fas ligand (Fas/FasL) pathways (Berke, 1989; Ramsay and Ramshaw, 1997).

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (and lymphokine-activated killer cells, LAK) are responsible for the granule-exocytosis pathway killing. This response is perforin dependent and involves the release

of several lysosomal enzymes and granules including perforin, granzymes, and proteoglycans. NK cells lack antigen specificity in their target recognition and lysis. Although NK cells can recognize major histocompatibility complexes (MHC) class I molecules on some antigen-presenting cells (APC), this recognition leads to inhibition of killing. In contrast, CTL recognize self-MHC class I ($CD8^+$) or class II ($CD4^+$) molecules on APC and their targets. This unique characteristic of the CTL and their ability recognize self-antigens ensure that invading intracellular pathogens are seen and contained by the immune system.

CTL are predominantly $CD3^+CD8^+$ cells, although $CD3^+CD4^+$ cells have been shown to have CTL properties, particularly via the Fas/FasL pathway. Many in vitro studies have shown that $CD4^+$ cells can become cytotoxic when antigens are presented via the MHC class II pathway or in the absence of $CD8^+$ cells. Indeed, $CD4^+$ virus-specific effectors have been reported in measles, herpes, and human immunodeficiency virus (HIV).

The role of CTL in HIV infection is very clear (Rowland-Jones et al., 1998a). Much data suggest that $CD8^+$ T cells are important in the control of HIV replication, especially early in the disease (Ada, 1996; Autran et al., 1996; Connick et al., 1996). Indeed, the lack of a CTL response presages a rapidly declining course of disease. Vigorous CTL responses have been reported in HIV-infected people with global impairment of immune functions whereas the absence of vigorous HIV-specific CTL in individuals with fairly good immune functions has also been reported (Bernard et al., 1998). This has raised questions as to the clinical relevance of CTL in HIV progression (Walker and Plata, 1990). We now know that HIV possesses the ability to escape CTL killing by mutation as well as other cellular evasion strategies. We shall discuss our current knowledge of the role of CTL in the control of HIV replication in this chapter.

ANALYSIS OF CTL

T-cell differentiation and selection in the thymus involves several stages (Fig. 6.1), culminating in the emergence of immature, differentiated, naive $CD3^+CD8^+$ and $CD3^+CD4^+$ cells bearing the αβ T-cell receptors. These naive cells are released into circulation and typically move between blood and the secondary lymphoid organs, particularly the lymph nodes. Encounters with antigen-presenting MHC class I or II cells, respectively, followed by costimulatory signals such as CD80/86, leads to the activation and development of a primary immune response as well as further differentiation of these cells into effector and memory cells (Mehta-Damani et al., 1994). This activation also requires the presence of IL-2, which is induced in a "positive feedback" manner, and, also, a corresponding up-regulation of the IL-2 receptors on the surfaces of these cells. The effector T cells are short-lived, usually lasting a few weeks. In contrast, the memory T cells, which differ from the naive T cells in

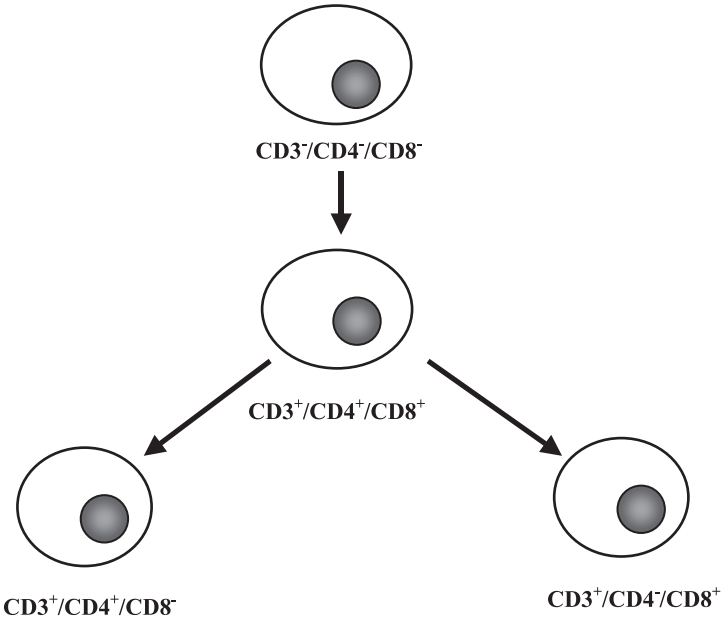

Figure 6.1. Positive selection of T-cells in the thymus.

that they express the CD45RO isoform and lack the need for costimulatory molecules, can remain in circulation at low levels for years (McMichael et al., 2000). The $CD3^+CD4^+$ cells typically differentiate into effector T-helper (Th)1 and Th2 cells based on the type of cytokine they secrete. Th1 cells secrete IFN-γ, IL-2, and tumor necrosis factor (TNF)-β, whereas Th2 cells secrete IL-10, transforming growth factor (TGF)-β, IL-4, and IL-5, although this is an oversimplification, and clones with a variety of cytokine secretion patterns have been reported. $CD3^+CD8^+$ cells typically differentiate into effector cytotoxic cells (CTL), which also secrete cytokines (Tc1 and Tc2), subject to the same conditions as $CD4^+$ T cells. The most effective APC include dendritic cells (DC), which are capable of picking up the pathogen (or proteins) and processing its protein to produce MHC-restricted, specific antigenic determinants (epitopes) that are recognized by the T cells. Thus, the presence of specific epitopes are essential for the development of both effector and memory T cells. Figure 6.2 shows a typical interaction between MHC-restricted, peptide-carrying APC and a T cell via the T-cell receptor. This interaction leads to the development of memory and effector T cells.

Most of the focus on CTL is based on the ability of these memory T cells to differentiate into effector cells on secondary encounters with the same antigens. This is the basis of most of our current vaccine strategies where pathogen-

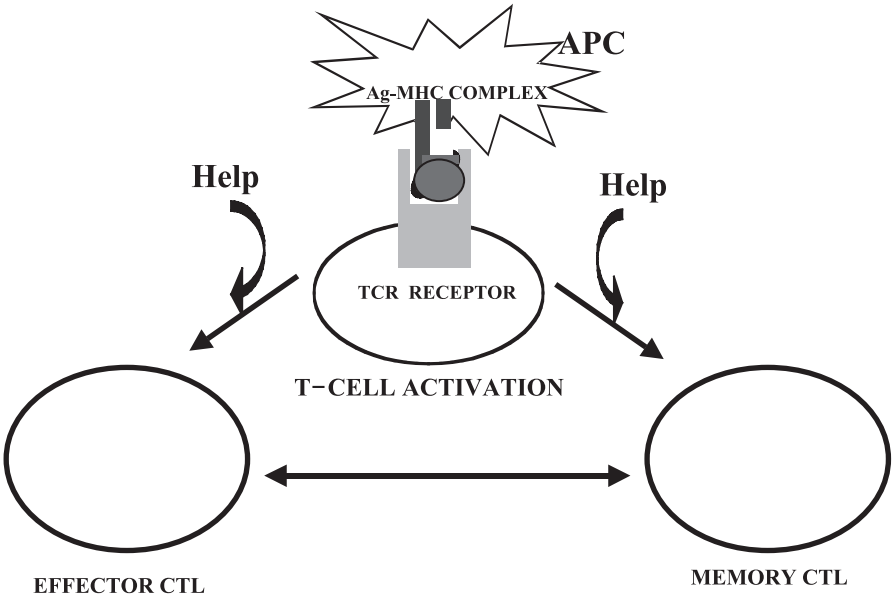

Figure 6.2. Generation of CTL.

specific antigens are introduced into the body in a nonpathogenic form with the subsequent development of memory T cells that are capable of developing into effector cells following secondary contact with the pathogen. In human studies, the presence of CTL in vivo can be seen by the presence of circulating in vitro restimulatable antigen-specific CTLs following most viral infections (including HIV) or vaccinations (Kourilsky et al., 1998; Schirmbeck et al., 1995).

HIV-specific CTL has been shown to exist in most individuals who have been exposed to the virus. These include children born to infected mothers, slow, fast, and nonprogressors, and HIV-exposed, uninfected individuals. The presence of these HIV-specific CTL have been shown through ex vivo studies involving freshly isolated peripheral blood mononuclear cells (PBMC), alveolar lymphocytes, lymph node biopsies, splenic lymphocytes, and lymphocytes derived from cerebrospinal fluid and vaginal secretions (Kaul et al., 2000; Quayle et al., 1998). A wide range of epitopes (8–11 amino acids) derived from every surface, structural and regulatory proteins of the HIV, specific for different human leukocyte antigens (HLA) have been described (Dadaglio et al., 1991). Shown in Table 6.1 are the defined HIV epitopes, which are restricted to a single HLA allele, HLA-A2. Even the one allele (of six usually expressed by an individual) shows a vast range of peptides, although not all are recognized by the individual (Betts et al., 2000).

TABLE 6.1. HLA Class 1-A2 Restricted CTL Epitopes Map

gp160

MRVKEKYQHLWRWGWRWGTMLLGMLMICSATE**KLWVTVYYGV**PVWKEATTTLFCASDAKAY
DTEVHNVWATHACVPTDPNPQEVVLVNVTENFNMWKNDMVEQM**HEDIISLWDQSLK**PCVKL
TPLCVSLKCTDLKNDTNTNSSSGRMIMEKGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIP
IDND**TTSYKLTSCNTSVITQACPK**VSFEPIPIHYCAPAGFAILKCNNKTFNGTGPCTNVST
VQCTHGIRPVVSTQLLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNT**SVEINCTRPNNNTRK**
RIRIQRGPGRAFVTIGKIGNMRQAHCNISRAKWNNTLKQIASKLREQFGNNKTIIFKQSSG
GD**PEIVTHSFNCGGEFFYCNST**QLFNSTWFNSTWSTEGSNNTEGSDTIT**LPCRIKQIINMW**
QKVGKAMYAPPISGQIRCSSNITGLLLTRDGGNSNNESEIFRPGGGDMRDNWRSELYKYKV
VKIEPLGVAPTKAKRRVVQREKRAVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGIV
QQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLLGIWGCSGKLICTTAVPW
NASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWN
WFNITN**WLWYIKLFIMIVGGL**VGLRIVFA**VLSIVNRV**RQGYSPLSFQTHLPTPRGPDRPEG
IEEEGGERDRDRSI**RLVNGSLALIW**DDLRSLCLFSYHRLRDLLLIVTRIVELLGRRGWEAL
KYWWN**LLQYWSQEL**KNSAVSLLNATAIAVAEGTDRVIEVVQGACRAIRHIPRRIRQGLERI
LL **855**

p17

MGARASVLSGGELDRWEKIRLRPGGKKKYKLKHIVWASRELERFAVNPGLLETSEGCRQIL
GQLQPSLQTGSEELR**SLYNTVATL**YCVHQRIEIKDTKEALDKIEEEQNKSKKKAQQAAADT
GHSNQVSQNY **130**

p24

PIVQNIQGQMVHQAISPR**TLNAWVKVV**EEKAFSPEVIPMFSALSEGATPQDLNTMLNTVG**G**
HQAAMQMLKETINEEAAEWDRVHPV**HAGPIAPGQMREPRG**SDIAGTTSTLQEQIGWMTNNP
PIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKN
WMTETLLVQNANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVL **230**

p2,p7,p1&p6

AEAMSQVTNSATIMMQRGNFRNQRKIVKCFNCGKEGHTARNCRAPRKKGCWKCG**KEGHQMK**
DCTERQANFLGKIWPSYKGRP**GNFLQSRPEPTAPPE**ESFRSGVETTTPPQKQEPIDKELYP
LTSLRSLFGNDPSSQ **130**

Protease

PQVTLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMSLPGRWKPKMIGGIGGFIKVRQYDQ
ILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF **90**

Reverse transcriptase (RT)

PI**SPIETVPVKL**KPGMDGPKVKQWPLTEEKIK**ALVEICTEM**EKEGKISKIGPENPYNTPVF
AIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVT**VLDVGDAYFSV**PLDE
DFR**KYTAFTIPSI**NNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPDI**VIYQY**
MDDLYVGSDLEIGQHRT**KIEELRQHLLRWGLTTPDKK**HQKEPPFLWMGYELHPDKWTVQPI
VLPEKDSWTVNDIQKLVGKLNWASQIYPGIKVRQLCKLLRGTKALTEVIPLTEEAELELAE
NRE**ILKEPVHGV**YYDPSKDLIAEIQKQGQGQWTYQIYQEPFKNLKTGKYARMRGAHTNDVK
QLTEAVQKITTESIVIWGKTPKFKLPIQKETWETWWTEYWQATWIPEWEFVNTP**PLVKLWY**
QLEKEPIVGAETFYVDGAANRETKLGKAGYVTNRGRQKVVTLTDTTNQKTELQAIYL**ALQD**
SGLEVNIVTDSQYALGIIQAQPDQSES**ELVNQIIEQL**IKKEKVYLAWVPAHKGIGGNEQVD
KLVSAGIRKVL **560**

TABLE 6.1. *(Continued)*

Integrase

FLDGIDKAQDEHEKYHSNWRAMASDFNLPPVVAKEIVASCDKCQLKGEAMHGQVDCSPGIW
QLDCTHLEGKVILVAVHVASGYIEAEVIPAETGQETAYFLLKLAGRWPVKTIHTDNGSNFT
GATVRAACWWAGIKQEFGIPYNPQSQGVVESMNKELKKIIGQVRDQAEHLKTAVQMAVFIH
NFKRKGGIGGYSAGERIVDIIATDIQTKELQKQITKIQNFRVYYRDSRNPLWKGPAK**LLWK**
GEGAVVIQDNSDIKVVPRRKAKIIRDYGKQMAGDDCVASRQDED **280**

Nef

MGGKWSKSSVIGWPTVRERMRRAEPAADRVGAASRDLEKHGAITSSNTAATNAACAWLEAQ
EEEEVGFPVTP**QVPLRPMTY**KAAV**DLSHFLKEKGGLEGL**IHSQRRQDILDLWIYHTQGYFP
DXQNYTPGPGVRY**PLTFGWCYKL**VPVEPDKIEEANKGENTSLLHPVSLHGMDDPERE**VLEW**
RFDSRLAFHHVARELHPEYFKNC **200**

Our current knowledge of HIV-specific CTL generation in vivo indicates that APCs play a crucial role (Knight et al., 1997; Plata et al., 1990). In heterosexual transmission, for example, it is believed that subepithelial dendritic cells play a role by picking, processing, and transporting macrophage-trophic virus across the genital epithelium and transferring the virus to activated $CD4^+$ T cells expressing the CCR5 coreceptor. Some immature dendritic cells are believed to carry the virus along the lymphatic routes (where they mature along the way) to the lymph nodes, where the virus is transferred to T cells (Cameron et al., 1996). Dendritic cells have the ability to activate some resting T cells, making them susceptible to HIV (Cameron et al., 1994; Zarling et al., 1999). Following this initial infection, the virus replicates in $CD4^+$ cells, causing infected-cell death and at the same time inducing both cellular and humoral immunity, which is usually evident by 3–6 months after infection. Macrophages also play a significant but less potent role in transferring HIV to T cells. Macrophages have an additional role of supporting HIV replication, mainly of the macrophage-trophic, nonsyncytium-inducing (NSI) viral subtype usually involved in initial infection. Although HIV-specific antibodies usually persist for life, CTL responses are variable over time, even within the same individual. We will discuss these changes and trends in the relevant sections below.

GENERATION OF CTL IN VITRO

Most of our understanding of CTL functions arose from in vitro studies, which are aimed at amplifying memory T cells by secondary stimulation using relevant antigens and autologous APCs (Zarling et al., 1999). Table 6.2 lists the most commonly used in vitro assays for $CD8^+$ cell function. These tests can be conveniently divided into measurements for constitutively secreted lymphokines, extent of fragmentation of target cell DNA, and studies of membrane integrity of target cells.

TABLE 6.2. Techniques for Measuring Cytokines

Lymphokine secretion:
1. Extracellular
   i. ELISA
   ii. Radioimmunoassay
   iii. Sensitive cell lines
   iv. ELISPOT
2. Intracellular
   i. Flow cytometry

DNA fragmentation:
1. JAM test
2. DNA quantification

Membrane stability:
1. ^{51}Chromium release
2. CARE-LASS (calcein-release-assay)
3. Time-resolved (BATDA)
4. Intracellular protein

Lymphokine Secretion Studies

HIV infection has been shown to lead to a variety of disturbances in the regulation of cytokine expression. These disturbances include a general increase in the expression of proinflammatory cytokines (TNF-α, IL-1, and IL-6) and antiviral IFN-α and -β, an increase in type 2 T-helper cytokines (IL-4, IL-10, and TGF-β), and a decrease in type 1 T-helper cytokines (IL-2, IL-12, IL-15, and IFN-γ) (Fan et al., 1993). These changes may play a role in the pathogenesis and dynamics of HIV infection (Bailer et al., 1999; Valdez and Lederman, 1997). TGF-β has been shown to be immunosuppressive and is up-regulated by the Tat protein of HIV (Reinhold et al., 1999). TGF-β up-regulation has been postulated to lead to immunosuppression of both B- and T-lymphocyte function, including inhibition of other cytokines such as IFN-α and -γ (Sharma et al., 1995). Our own studies suggest that HIV-specific TGF-β may be one cause of nonspecific immunosuppression in HIV infection.

Many studies of cytokines have used IFN-γ as a surrogate marker for CTL function. IFN-γ is produced by NK cells and T lymphocytes. IFN-γ induces the synthesis of host proteins important in defense against intracellular pathogens. These include MHC class I and II molecules, TNF-α, adhesion molecules, NADPH oxidase, and nitric oxide. IFN-γ also down-regulates Th2 responses in addition to having a modest direct antiviral activity (Valdez and Lederman, 1997).

Measurement of IFN-γ typically involves in vitro bulk studies that use restimulation of T cells with HIV-specific peptides, proteins, or recombinant viruses. IFN-γ is measured in a variety of ways. These include techniques for measuring secreted IFN-γ that range from enzyme-linked immunosorbant

assays (ELISA), radioimmunoassay, or IFN-γ-sensitive cell lines, which measure secreted IFN-γ in culture supernatants, to the ELISPOT assay, which uses an immobilized, high-protein binding, anti-IFN-γ antibody-coated membrane to capture secreted IFN-γ as it is secreted (Larsson et al., 1999). The number of cells secreting IFN-γ is then determined by adding biotinylated secondary capture antibody followed by the use of avidin-bound biotinylated horseradish peroxidase and color reagent. The number of spots counted indicates the number of cells producing INF-γ, and HIV-specific spots are determined by subtracting the number of spots in control, nonstimulated culture wells.

More recently, focus is shifting toward measuring intracellular IFN-γ and other cytokine levels using flow cytometry (Prussin, 1997; Prussin and Metcalfe, 1995; Vikingsson et al., 1994). This has the advantage of giving more information on the cell types producing the cytokines, and other markers such as activation or (CD69, CD25) apoptosis (7AAD, propridium iodide) can be measured at the same time. The technique involves stimulation of whole blood or isolated PBMC with peptides or recombinant viruses, mainly vaccinia. This stimulation could be done for a short or long duration depending on the methods used. For example, peptide stimulation is usually done for a period of 4–6 h in the presence of costimulatory molecules (anti-CD28 and anti-CD49d), whereas viral stimulation is usually done for 18–24 h corresponding with the periods of peak cytokine productions. Irrespective of the method of stimulation used, within the last 4 h of stimulation, secretion of cytokines from the cells is inhibited by using monensin or brefeldin A, which, act to prevent the exocytosis of produced cytokines. This step is followed by the fixing and permeabilization of the cells followed by staining with relevant conjugated antibodies specific for surface and intracellular molecules. Cellular analysis is then performed using flow cytometry (Yamamura et al., 1995). Figure 6.3 shows an example from our lab where $CD3^+/CD8^+$ cells were gated and the proportion of these cells that are $CD69^+$ and produce IFN-γ are calculated.

The main disadvantage of all these methods is that they do not measure direct cellular killing but, rather, indicate the ability of these cells to recognize and act against HIV-specific antigens and produce cytokines.

Measurements of DNA Fragments

These studies rely on the ability of DNA to break down into fragments during cellular apoptosis. Within minutes of CTL target cell contact, nuclear damage leading to double-stranded DNA break followed by cleavage of DNA into small oligonucleotides is noted (Duke, et al., 1988). This step is important in the ultimate cell lysis and the extent of this damage has been shown to correspond to the efficiency of CTL lysis of the cells. DNA fragmentation is not peculiar to CTL lysis, and other causes of cell death have the same consequence. The method essentially involves the collection of released DNA fragments using a DNA harvester or detergent lysates and quantifying them. The data derived has

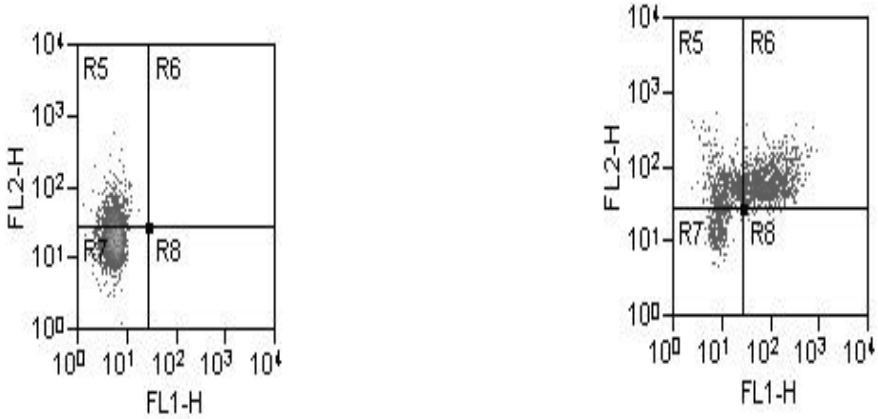

Figure 6.3. Intracellular Cytokine Assay.

been shown to generally correlate with CTL lysis. A related method is the JAM test where radioactive labels like [^{3}H]TdR, are used to label the target cells. The amount of intact DNA retained by living cells is then measured by harvesting the cells as in proliferation assay and measuring the amount of radiation incorporated into the intact DNA (fragmented DNA from dead cells is washed through the filters and lost) using radioactive counters (Matzinger, 1991). The drawback of these techniques is related to the differences in the DNA fragmentation in target cells from the same and different subjects, the dependence of this mechanism on the actual number of effector cells used, and nonlinearity of CTL killing (Ratner and Clark, 1991). In addition, some cell types such as resting fibroblasts have been shown to spontaneously release DNA during CTL killing which may affect the data.

Membrane Stability

These tests are aimed at measuring a breach in the plasma membrane of target cells as a consequence of CTL killing. The hallmark of CTL killing is the lysis of the target cells by either the perforin-dependent granule-exocytosis pathway (Koopman et al., 1997) or the Fas/FasL pathways. This lysis leads to

the breakdown of the cellular organelles and cell membrane and ultimately cell death.

The granule-exocytosis pathway involves tight, calcium-independent, effector/target adhesions through the T-cell receptor-MHC/peptide molecules. Priming and triggering of cell killing is calcium dependent. The next step is the release of granules from the "cores," including perforin and a number of granzymes, particularly granzymes A and B (Spaeny-Dekking et al., 1998). These granules act on the cellular membrane of the target cells, leading to a break in the membrane and cell death.

The Fas/FasL pathway is independent of perforin and other granules (Kajino et al., 1998). It is also triggered by the trimodal cross-linking of TCR/MHC/antigen. It involves the cross-linking of the Fas ligand expressed on the surface of the effector CTL and the Fas molecule expressed on the surface of the target cells, leading to a triggering of a cascade of mechanisms culminating in apoptotic target cell death.

The most used method of measuring membrane integrity is the chromium release assay (Segal and Snider 1989). In this assay, target cells expressing MHC-restricted antigens are pulsed with radioactive ^{51}Cr. The cells are then plated in culture with autologous effector CTL, either freshly isolated or following in vitro expansion. The CTL are added at different ratios to the target cells, usually from 100:1 to 3.125:1. Target cells expressing an irrelevant antigen are used as control in parallel wells. The culture period used is usually 4–6 h. The encounter between the target and the CTL effectors should commence killing of the target within minutes of this incubation, leading to the release of the chromium into the culture media. At the end of the incubation, the supernatents are collected, and the amount of chromium released is measured using a gamma counter, which measures the released ^{51}Cr. The data is then analyzed by calculating the percentage of specific killing or lytic units. Percentage of specific killing is calculated using the formula:

$$\frac{\text{amount of experimental chromium released} - \text{spontaneously released chromium} \times 100}{\text{amount of maximum chromium released} - \text{spontaneously released chromium}}$$

The spontaneous chromium released refers to the wells containing only target cells, whereas the maximum release data refer to wells containing target cells and detergents, such as 2.5% Triton, which has the capability of lysing all the cells and releasing maximum amount of chromium. The antigen-specific CTL killing is then calculated for each E:T ratio.

Other less routinely used studies of membrane integrity include non-radioactive techniques such as the CARE-LASS, a fluorometric microassay (Lichtenfels et al., 1994). In this assay, an acetoxymethyl ester of calcein, which crosses cell membrane easily, is added to the target cells and is converted to the polar fluorochrome calcein in the cytoplasm of intact cells. In the presence of CTL killing, this fluorochrome is released and measured using an automated fluorescence scanner. Another closely related technique is the time-resolved

fluorometric assay, which uses different esters (BATDA) and a chelator (Eu^{3+}) (Blomberg et al., 1996). Measurement of quantifiable, released intracellular protein has also been described as a method of measuring CTL killing (Kyburz et al., 1993).

CTL in Primary HIV Infection

Primary HIV infection represents the period immediately post infection with the HIV virus, which is characterized by a number of immune changes and nonspecific clinical symptoms such as rash, fever, and lymphadenopathy, which, for the most part, resolves spontaneously, leading to a long asymptomatic period. The patient is characterized with lack of HIV antibodies and high levels of plasma HIV-1 RNA that subsequently declines and reaches a quasi-steady state after about 6 months (Musey et al., 1997b). Whereas antibody is detectable at about 3–6 months following infection, HIV-specific CTL have been found in early primary infection period corresponding with the time of the initial decline in viremia (Wilson et al., 2000). The emergence of vigorous, HIV-specific CTL in most individuals has been implicated as a reason for the declining viremia (Price et al., 1999; Shankar et al., 1999).

In several cross-sectional and prospective patient studies, a wide range of CTL responses have been reported. In one study, fresh PBMC from 23 patients (followed for 2 years following primary infection) were tested for the presence of HIV-specific CTL. Most (74%) of the donor PBMC contained HIV-specific CTL at a range of 10–26% above control vaccinia (at 100:1 E:T ratio) using the chromium release assay. HIV-1 *env* gene product was recognized by 94% of the patients whereas 29% and 12% of the patients recognized HIV-1 Gag and Pol, respectively. The same study also found that HIV-specific CTL were mostly $CD8^+$ cells, although 22% of the patients also had $CD4^+$ CTL. These findings were found to be similar when the patients were studied at intervals of 3–6 months (Musey et al., 1997).

Memory T cells (as measured by limiting dilution assay, or LDA) are also induced during this period and they reach their peak levels during primary infection followed by subsequent decline. Most responses have equally been targeted at the HIV-1 *env* gene products and, to a lesser extent, the HIV-1 *gag* gene products. The precursor frequencies per million PBMC in one study were found to range from 0 to 446 for Env, 0 to 333 for Gag, and 0 to 364 for Pol gene products. With the advent of direct enumeration by tetramers, these are thought to be low estimates by as much as two orders of magnitude (Mylin et al., 2000). These frequencies were, however, found to be variable in the same individual when followed over time. The HIV-1 Env-specific memory CTL responses where found to be inversely correlated with viral load and plasma level of infectious virus, whereas the HIV-1 Gag-specific memory CTL responses inversely correlated with plasma levels of infectious virus and not viral load. This and similar studies indicate that the *env* gene product is the most dominant gene product involved in the control of viral replication (Musey et al., 1997).

During primary infection, the presence of strong HIV-specific CTL has also been found to correlate with a high number of $CD4^+$ cell counts. This correlation was more marked with the presence of *env*-specific CTL. Those patients with an initially high *env*-specific CTL tend to progress more slowly than those with relatively lower Env-specific CTL in the beginning of their infection (Musey et al., 1997).

Despite these strong findings and correlation of CTL and control of viral replication, nearly all individuals ultimately develop acquired immunodeficiency syndrome (AIDS) within a decade if untreated. This has led to skepticism as to the value of in vitro measured CTL as it relates to in vivo functions. It was, however, found that HIV can evade CTL killing by several mechanisms (Price et al., 1997). One mechanism is the presence of naturally occurring HIV-1 epitopes that impair CTL recognition by induction of anergy. These are naturally occurring, spontaneous mutations that may or may not play a significant role in viral escape as the evidence is not conclusive. Other spontaneous factors described included the lack of proofreading capacity of the HIV-1 replicases, which, coupled with the high viral replication, could lead to a number of chance mutations that lead to the emergence of fresh epitopes, unknown to the memory CTL (or unable to bind MHC) thereby escaping recognition and lysis (Price et al., 1997; Soudeyns and Pantaleo, 1997).

Studies of adaptive evolution of HIV-1 in the host environment have given more insight into viral escape mechanisms. These studies were done with due consideration of the extreme genetic instability of the HIV-1 virus. One such study focused on the period of the most vigorous CTL responses during primary infection. The study looked at five HIV-infected individuals with an HLA B8 allele. Early in the infection, a strong CTL response to an HLA B8-restricted epitope (FLKEGGL) that was derived from residues 90–97 of the HIV-1 (HIV-LAI) Nef protein was seen (Price et al., 1997). One of these patients was followed for 500 days without antiretroviral therapy (deferred treatment) and within 22 days following the onset of symptoms, several positively selected mutations of this epitope that were suboptimal ligands for autologous CTL were noted. These mutations increased with time and essentially overgrew the original virus thereby demonstrating escape from CTL lysis.

CTL IN POSTPRIMARY INFECTION

This period could last as long as 10–20 years without treatment. The patient can be asymptomatic for many years or be symptomatic as early as 2 years postinfection. The main characteristic of this period is the gradual loss of $CD4^+$ cells and corresponding increase in $CD8^+$ cells leading to the reversal of $CD4^+/CD8^+$ ratio. This period also represents the period of gradual failing of the immune system and the appearance of opportunistic infections (Miedema and Klein, 1996). The period ends with the development of AIDS ($CD4^+ < 200/\mu L$) and subsequent death of the patient. Treatment with highly active

antiretroviral therapy (HAART) has changed both the morbidity and mortality during this period and some of the immune deficiencies have been reversed. Most opportunistic infections have disappeared since the introduction of HAART.

The postprimary infection period is a time of high variability in HIV-specific CTL even within a patient (Gamberg et al., 1999). After the attainment of a quasi-steady state in both viral load and CTL late in primary infection, the expectation here is that CTL and other immune parameters would maintain good control of viral replication. The picture, however, is that of failing immune containment and a decline in absolute and relative $CD4^+$ cells following post-infection lysis of target cells. Viral replication is ongoing at a very high rate (Perelson et al., 1997). This period is also dominated by the emergence of the synctitial-inducing (SI) virus, which utilizes a different set of coreceptors (CXCR4) than the NSI virus involved in the initial primary infection (CCR5 coreceptors). This virus is T-cell trophic and its appearance, in most part, correlates with advancing HIV disease.

During this period, diverse epitopes are usually available and CTL responses may be less effective as a result of the escape mutations taking place (Miedema and Klein, 1996). The depletion of $CD4^+$ T cells caused by the lysis of virus-infected cells also deprives the system of T-helper activity. This has been suggested as a cause of the failure of CTL to function optimally. Most recently, we found that a subset of individuals in this period produce TGF-β that accounted for nonspecific inhibition of IFN-γ production by $CD8^+$ cells.

CTL responses during this period are directed at all of the viral proteins. Specific epitopes for the Env, Gag, Pol, Rev, and Nef proteins have been described (Gotch, Gallimore et al., 1996; Haas, Samri et al., 1998). Unlike the primary infection period where the dominant CTL response is directed at the Env protein, this period is characterized by a wide range of responses among individuals and within an individual. CTL response against the Env protein is complicated by the presence of extensive sequence variations arising from escape mutations and also by the presence of non-MHC-restricted cell lysis found to be Env-specific. Some studies have shown that Env-specific lysis can actually be inhibited by the use of anti-CD3 and, to some extent, anti-CD8. Further studies have concluded that both MHC-restricted CTL and antibody-dependent cell-mediated cytotoxicity (ADCC) are involved in Env-specific cell lysis. The ADCC-mediated killing involved $CD16^+$ cells armed with Env-specific cytophilic antibodies. Several overlapping epitopes involving the gp41 and gp120 regions have been described (Kalams and Walker, 1994).

Most studies however, have shown that the most potent response is seen against the highly conserved HIV-1 *gag* gene product at this stage (Johnson et al., 1991). Indeed, one study involving slow and fast progressors to AIDS has shown that strong Gag-specific CTL in conjunction with lower HIV-infected $CD4^+$ cells correlates with slow but not fast progression to AIDS (Kalams and Walker, 1994). Gag is a p55 protein that is cleaved to four other subunits—p6, p7, p17, and p24. Most of the Gag-specific CTL response is directed at the

p24 subunit, which is raised substantially during early viral replication (Klein et al., 1995). This region presents several clusters of class I restricted epitopes that are recognizable by the CTL. HIV-specific CTL is also seen among the most conserved of the HIV proteins—the polymerase protein (Pol). It is also found to be HLA class I restricted and several epitopes have been described. HIV-specific CTL against the regulatory Nef proteins has been described (Bauer et al., 1997). It is an early expressed protein and a factor involved in viral replication that is implicated as a cause of MHC class I down-regulation leading to viral escape of CTL killing (Cohen et al., 1999; Collins et al., 1998). The presence of Nef-specific CTL is seen as good sign for possible control of viral replication by the CTL. Moreover, Nef-specific epitopes were found to be restricted by several HLA molecules, making it a good target for a wide variety of CTL from different individuals (Kalams and Walker, 1994).

HIV-specific CTL has also been implicated as a part of pathogenesis of HIV disease and the development of AIDS. Most studies have shown that the development of AIDS coincides with the time of deteriorating CTL responses in HIV-infected individuals, giving a picture of a possible protective role being played by the CTL in slowing down disease progression (Goulder et al., 1997). In the same vein, some studies have implicated the CTLs as the cause of pathology seen in some of the organs they infiltrate, notably the lungs and the brain (Krakauer and Nowak, 1999; Wodarz and Krakauer, 2000). HIV-specific CTL has been isolated from both sites (Plata et al., 1990). Whereas interaction between $CD44^+$, non-ADCC-mediated, HIV-specific CTL and local alveolar macrophages is thought to lead to localized inflammatory reactions and subsequent functional abnormalities of the lung parenchyma, the development of AIDS dementia complex is more glaring (Twigg et al., 1999). The cerebrospinal fluid (CSF) and brain tissues of HIV-infected individuals with this disorder are known to be infiltrated by $CD8^+$ lymphocytes with CTL capabilities. This suggests that CTL may have a role in the pathogenesis of AIDS dementia complex through direct or indirect cytotoxicity (Kalams and Walker, 1994).

The HIV pathogenesis is a continuous spectrum that ends with the development of AIDS and the death of the patient. The most marked feature of the AIDS period is the presence of several opportunistic infections from pathogens that are normally not a problem for a normal immune system. The decline in health status is also accompanied by a decline in all immune parameters including CTL. Several mechanisms have been advanced as the cause of the decline in the HIV-specific CTL but no explanation is conclusive (Goulder and Walker, 1999; Soudeyns and Pantaleo, 1997; Zerhouni et al., 1997). Some studies have suggested that escape mutations, down-regulation of MHC class I, decreased expression of surface markers such as leukocyte function-associated antigen (LFA-1), defects in APC and CTL clonal expansion/exhaustion leading to anergy are responsible for this decline in effective CTL response (Lewis et al., 1994; Wodarz et al., 1998). Indeed, one study has shown that the slow progressors, unlike the rapid progressors, accumulate far more nonsynonymous mutations (involving amino acid changes) than synonymous mutations (Birk et

al., 1998). This correlates with the presence of stronger CTL responses in the slow progressors as compared with the rapid progressors, thereby indicating the possibility of selective pressure being exerted by the CTL. Other factors suggested included the presence of secreted molecules that impair CTL responses. Earlier studies have shown that the *tat* gene product of HIV is capable of causing immunosuppression through the induction of TGF-β (Reinhold et al., 1999), although our experiments show that TGF-β production can be mediated by responses to HIV independent of Tat. It also induces changes in cells derived from neural tissues and lymphoid organs (Jurkat cells).

CTL IN HIV-INFECTED INFANTS

Perinatally infected children also show differential progression and pattern of HIV pathogenesis. Without treatment, 20% of them develop full-blown AIDS within the first year, whereas most of the rest develop AIDS and die within the first 5 years. Few children go on to develop adult-like disease pattern with slow progression to AIDS.

The neonatal period is characterized by the abundance of immature lymphoid cells including T cells (Delespesse et al., 1998). The functions of neonatal T cells have also been reported as less effective than their adult counterparts. However, some studies have indicated that neonatal T cells are capable of mounting significant viral-specific responses. Indeed, a series of studies of newborns that are perinatally infected with HIV has shown the presence of strong HIV-specific CTL directed against the Gag, Pol, and Env proteins (Buseyne et al., 1998a; Pikora et al., 1997). CTL has been demonstrated in both ex vivo and in vitro stimulated lymphocyte studies and it was found to be HLA class I restricted. The in vitro CTLs seen in this group were the same as those of an adult population, whereas the ex vivo CTLs were reported as slightly lower than those of an adult population. One such study has shown that the presence of strong HIV-specific CTL correlated with children who did not progress to AIDS within the first year of birth, whereas those who progressed to AIDS within the first year had lower CTL responses. Although viral load and T-cell numbers were unrelated to the CTL levels in the first 6 months, they were found to be related between 7 and 12 months, with lower viral load and higher number of both $CD4^+$ and $CD8^+$ cells indicating a possible beneficial role of CTL in these infants (Riviere and Buseyne, 1998).

CTL IN HIV-EXPOSED SERONEGATIVE INDIVIDUALS

The exposed seronegative individuals represent a group of individuals who are repeatedly exposed to the HIV virus but have presumably failed to be infected. They present an interesting group as they provide a hope for the presence of a host defense mechanism against the HIV virus that can be exploited in the

search for vaccines. Several cohorts of these individuals exist, including men who have sex with men, intravenous drug users, commercial sex workers, exposed healthcare workers, heterosexual couples in monogamous relationships, and children born to infected mothers (Rowland-Jones et al., 1993, 1998b). In almost all these groups, HIV-specific CTL has been shown to exist, although the nature and significance of these responses differ from one study to another. In a study of exposed seronegative commercial sex workers in Gambia and Kenya, for example, 6 out of 15 Kenyan and 5 out of 6 Gambian sex workers showed HIV-specific CTL following repeated testing (Rowland-Jones et al., 1995). In our study of a cohort of 47 Zambian exposed-uninfected individuals who are involved in monogamous relationships with HIV-infected individuals, 9 individuals (20%) elicited HIV-specific CTL following a single assay using the chromium release assay. We also found that the presence of HIV-specific CTL in these individuals correlates with high viral load in their corresponding HIV-infected partners. It is, however, not clear whether these results represent what actually happens in vivo or whether they definitely play a role in preventing these individuals from getting infected.

CTL IN VACCINE DEVELOPMENT

It is widely believed that the only way to slow down the HIV epidemic is through the use of an effective vaccine. This view is bolstered by the findings showing a possible role being played by HIV-specific CTL in slowing down the progression to HIV in several studies. Several candidate vaccines have been developed and more are in development (Girard et al., 1999; Gorse et al., 1999). Most of the initial candidate vaccines were based on recombinant envelop proteins and were targeted at obtaining neutralizing antibodies to HIV (Johnston, 1997). The frequent mutations involving the *env* gene segment and the several findings that CTL might play a role in control of HIV lead many to conclude that an effective vaccine must have the capability of eliciting both humoral and cellular immunity (Kourilsky et al., 1998). There is a lot of skepticism regarding the finding of an effective HIV vaccine because of the high genetic variation of HIV quasi-species, even within the same individual. It is believed that any effective vaccine must be able to have polytypic properties that will cover as many HIV quasi-species as possible (Woodberry et al., 1999). There is also a concern raised regarding the ability of the virus to enter a latency stage in some cells thereby escaping the immune system as well as the need for vaccines that will confer persistent immunity to control the virus. Most of the current data involving vaccines are from animal studies. Although most murine and some primate studies have shown mild to strong HIV-specific CTL following immunizations with candidate vaccines, it is unclear whether that will be the picture in humans.

Using canary pox vaccine, one group has demonstrated the presence of HIV-specific $CD8^+$ CTL responses in seronegative volunteers (Belshe et al., 1998; Evans et al., 1999). Other studies have shown significant CTL responses of

seronegative volunteers vaccinated with a combination vaccination regimen involving different viral envelops (Corey et al., 1998) and even cross-clade CTL activities following vaccination (Ferrari et al., 1997).

It is generally believed that live attenuated vaccines will offer the best hope for inducing CTL (Bangham and Phillips, 1997). However, this is risky for several reasons, particularly the fear that attenuated HIV might not be safe enough. The next best approach has been the use of other recombinant viruses that can produce HIV gene products but with lower chance of causing morbidity or mortality in humans (Rolph and Ramshaw, 1997). So far, vectors such as attenuated vaccinia viruses, alpha viruses such as Venezuela equine encephalitis virus (VEE), and pox viruses are being used (Gorse et al., 1999). There is insufficient data to conclude whether CTL must play a role in these vaccines. Other approaches used include the development of DNA vaccines, live attenuated bacterial vaccines, immunostimulatory complexes, otherwise known as ISCOMS, and, to some extent, development of liposomal delivery systems that deliver soluble antigens for processing and incorporation into the MHC class I/peptide complex. None of these methods has so far yielded any data regarding the role of CTL in disease prevention.

CROSS-CLADE HIV-SPECIFIC CTL

One of the most remarkable effects of HIV infection is the viral diversity seen within and between people, places, and regions, which has raised some skepticism about finding a globally effective vaccine. Recent findings by our lab and others have however shown that HIV-specific CTL directed against one clade of the virus can actually kill viruses of other clades (Betts et al., 1997; Buseyne et al., 1998b; Jolly et al., 1992). In our study, for example, we examined HIV-specific CTL activity of eight Zambian subjects infected with a clade C virus as confirmed by heteroduplex analysis and partial sequence analysis of *env* and *gag* genes. Six out of the eight individuals studied showed strong CTL activity against recombinant vaccinia virus-infected targets expressing HIV Gag, Pol, and Env proteins derived from clade B HIV-1 (IIIB). Other labs have found similar results. Another interesting finding is the presence of shared epitopes between HIV-1 and HIV-2, which is less aggressive than HIV-1 and less widespread but more predominant in some parts of the world (Nixon et al., 1990). This might give hope for a possible pan-HIV vaccine. Some of these epitopes are also shared between individuals with different HLA subtypes (Lynch et al., 1998).

TREATMENT AND CTL DYNAMICS

Several antiretroviral drugs have been developed over the last decade. Whereas most of the earlier drugs used in monotherapy have been minimally effective, the use of combination drug therapy has proven to be very effective. Highly

HAART is the classical term describing the modern-day combination of antiretroviral agents that attack the virus at different stages of development and at different viral structural or regulatory levels. This has dramatically reduced morbidity and mortality from HIV and has further changed the general pathogenesis of the HIV disease in developed countries (Oxenius et al., 2000; Soudeyns et al., 2000).

The effects of HAART on CTL responses have been studied in some detail, giving rise to two different viewpoints. Some data has suggested that the potency of HIV-specific CTL declines with initiation of and complete viral suppression by HAART. This view is explained, in part, by the need for viral antigens to sustain the presence of strong CTL responses. The reduction of viral burden will, therefore, lower the amount of available antigens with consequent decline in HIV-specific CTL responses (Kalams et al., 1999). A second group of data suggests that strong CTL responses are found after viral suppression with HAART and subsequent HAART discontinuation. These data further showed that HIV-specific CTL correlate with strong post-HAART suppression of viraemia for up to 2 years (Ortiz et al., 1999). In a similar study at our laboratory, we found that treatment with protease inhibitors (and not nucleoside analogs), positively correlated with HIV-specific IFN-γ response by $CD8^+$ cells. This and similar studies suggest that indeed HAART improves some aspect of immune recovery including CTL. It is not yet clear why some individuals on HAART do have lower responses than others. Perhaps other unidentified factors may be responsible for these differences in responses by different individuals.

EFFECTS OF CTL ON PLASMA VIRAL LOAD

The level of HIV viral burden has been used as one of the yardsticks for monitoring the progression of an individual to AIDS. A lower viral load correlates with better clinical status and a higher viral load represents a worsening state of the disease. As described in previous sections, HIV-specific CTL has been shown to positively correlate with some level of protection against the progression of HIV-infected individuals to AIDS. It is therefore interesting to note the relationship between viral load and CTL responses in HIV-infected individuals.

Information currently available indicates that there is a strong correlation between viral load and CTL responses in different groups of individuals (Ariyoshi et al., 1995; Klenerman et al., 1996). Some have found both negative and positive correlations between viral load lowered by drugs (see above) and CTL responses (Borrow et al., 1994; Koup et al., 1994). Most still conclude that there is an inverse correlation. In another study carried out in our laboratory, 17 HIV-infected long-term survivors, who have remained healthy for 10+ years despite varying levels of both $CD4^+$ T cells and viral burden, were followed up for 10–14 years (Betts et al., 1999). Changes in both their viral load and CTL activities were measured and compared over time. Figure 6.4 shows the inverse correlation that existed between Pol-specific CTL and the viral load.

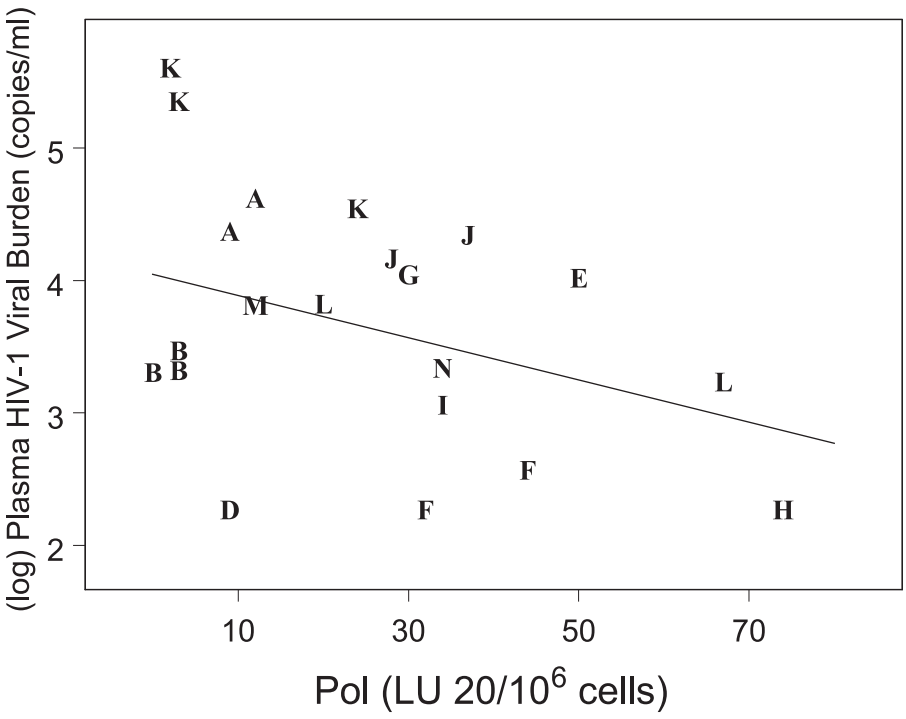

Figure 6.4. Viral load/CTL correlation.

The data also showed that the combined effects of both Pol- and Env-specific CTL are a stronger predictor of HIV-1 viral load burden than the effects of Pol-specific CTL alone. This clearly suggests that CTL may have played a role in the long-term survival of these individuals by keeping their viral replication in check.

CONCLUSION

HIV-specific CTL responses are complex and depend on several factors, including viral mutations, viral load, host factors such as disease progression, stage of infection, other immune regulatory factors, and drug use. It also depends on whether the patient is an infant or adult. Our current knowledge of these HIV-specific responses has largely been obtained by ex vivo and in vitro studies.

In general, strong HIV-specific CTL has positively correlated with a decline or slowing of disease progression as well as a state of better well being in the individual. The development of AIDS has been linked to a drop in effective CTL responses. Despite these findings, however, it is still not clear whether HIV-specific CTL play a significant role in containing HIV infection in vivo and how such an effect is mediated.

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7

Analysis of the α/β T-Cell Receptor Repertoire in HIV Infection

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INTRODUCTION

Antigens are presented to the immune system in the form of short peptides associated with class I or class II major histocompatibility complex (MHC) components. Recognition of MHC-peptide complexes is mediated by the T-cell receptor (TCR), a disulfide-linked heterodimer expressed at the surface of most thymocytes and peripheral T cells. TCRs composed of γ and δ chains ("γ/δ TCRs") are expressed by small subsets of peripheral and tissue-resident lymphocytes, and recognize soluble or tissue-associated antigens, including non-peptidic components of bacterial cell walls (Kaufmann, 1996; Tanaka et al., 1995). α/β TCRs recognize class I and class II peptides-MHC complexes (Davis and Bjorkman, 1988; Yague et al., 1985). The TCR is expressed at the cell surface in association with a set of invariant molecules collectively referred to as CD3 (Cosson et al., 1991). Upon cognate interaction between the TCR and peptide-MHC, complex supramolecular clustering is followed by conformational changes in the TCR and CD3 complex, resulting in the transduction of transmembrane activation signals to the T cell (Grakoui et al., 1999; Weiss and Littman, 1994). T-cell activation leads to increase in cell size and metabolism, enhancement of cell-surface expression of TCR and coreceptors, and expression

of differentiated function such as cytotoxic activity and production of cytokines (Cantrell, 1996).

THE T-CELL RECEPTOR

Rearrangement, Diversity, and Genetics

TCRs are highly polymorphic. The generation of primary sequence diversity in mature TCR genes results from a nonconservative somatic recombination process taking place between interspersed variable (V), diversity (D), and joining (J) TCR gene segments (Davis and Bjorkman, 1988). This process is highly analogous to that involved in the generation of antibody diversity (Tonegawa, 1983). Recombination is initiated at specific sites termed recombination signal sequences (RSS), which flank the gene segments to be rearranged and are specifically targeted by the RAG1–RAG2 recombinase (Ramsden et al., 1997; Schatz et al., 1989; van Gent et al., 1996). During this process, coding ends are differentially processed by exonuclease and terminal deoxynucleotidyl transferase, leading to the generation of tremendous primary DNA sequence diversity at the V(D)J interface (Gilfillan et al., 1993; Komori et al., 1993). Coding and signal joints are then relegated while intervening stretches of DNA are lost (Lewis, 1994). TCR rearrangement occurs during thymocyte maturation. Functional rearrangement of the TCR β chain prevents recombination of the second β chain allele, which often remains in germ-line configuration. α Chain rearrangement then proceeds, but allelic exclusion is weaker than with the β chain (Malissen et al., 1988, 1992). Hence, a proportion ($> 30\%$) of peripheral T cells expresses two functional TCR α chains, but the physiological significance of this phenomenon is unclear.

Over the years, at least 47 different functionally expressed TCR β chain variable region (TCRBV) genes and a large number of alleles were described, largely based on studies of cDNA and genomic libraries (reviewed in Arden et al., 1995; Wei et al., 1994). These genes were grouped into 34 families and a number of subfamilies based on nucleotide sequence relatedness, laying the foundation for the TCR β chain gene nomenclature currently in use (WHO-IUIS, 1995). Two functional TCRBD, 13 TCRBJ, 42 TCRAV, and over 60 different TCRAJ gene segments were similarly described (Arden et al., 1995; Yoshikai et al., 1985). The majority of TCR V-region genes show a large degree of allelic polymorphism, resulting in a very large number of TCR V-region haplotypes segregating within the general population (Cornelis et al., 1993; Posnett et al., 1986). The TCR β chain locus occupies 685 kb of DNA on human chromosome 7, where it comprises eight modular blocks of divergent homology units encoding ordered assemblies of TCRBV genes (Rowen et al., 1996). This suggests that the TCR loci have evolved through replicative duplication of an ancestral prototypic V gene subunit. Indeed, the β chain locus is rich in repetitive DNA derived from various classes of transposable elements,

which could confer a degree of genetic flexibility consistent with the diversity and polymorphism of the β chain locus (Smit and Riggs, 1996).

Structure and Function

TCR components are members of the immunoglobulin (Ig) gene superfamily. As with Igs, alignment of TCR genes reveals "framework" regions of relative homology interspersed with three clusters of DNA sequence diversity (complementarity-determining regions, CDR1, 2, and 3). The TCR β chain contains a fourth variable segment (CDR4), located between CDR2 and CDR3. Early models and functional mutagenesis data predicted that the CDRs were exposed to solvent, forming a large hypervariable surface containing the site involved in contacting the antigen-MHC complex (Chothia, 1988; Jorgensen et al., 1992; White et al., 1993). Since then, this representation was largely confirmed by X-ray crystallography (Bentley et al., 1995; Fields et al., 1995; Garboczi et al., 1996; Garcia et al., 1998; Reinherz et al., 1999). CDR3 is the most polymorphic TCR domain and the one most directly involved with antigenic peptide recognition. Three key structural features of CDR3 predominantly contribute to this interaction: a) the amino-acid sequence of CDR3, which is critical for physical recognition of the antigenic peptide; b) CDR3 length, which influences how deep the loop can reach into the peptide-binding groove of the MHC molecule and represents the first structural feature to be fixed during the maturation of antigen-specific immune responses (McHeyzer-Williams and Davis, 1995; Rock et al., 1994); and c) the identity of the J segment used in TCR rearrangement, inasmuch as germ-line J region contributes at least four polymorphic residues to the C-terminal portion of CDR3. As will be discussed below, the study of some of these structural features has revealed important characteristics of antigen-specific T cell-mediated immune responses.

The TCR Repertoire

As a general rule, each individual T-cell clone bears a unique cell-surface TCR α/β combination, corresponding to a defined antigenic specificity. The TCR repertoire is operationally defined as the sum of all TCR combinations and specificities expressed by peripheral T cells within a given host. The extent of TCR diversity that can be generated through the rearrangement process is astonishing: over 10^{15} different TCRs and combinations can be produced, sufficient in theory to enable the recognition of every conceivable peptide antigen (Davis and Bjorkman, 1988). The TCR repertoire is shaped during positive and negative thymic selection. That, as well as a number of molecular constraints, imposes practical limitations on the size and variability of the "mature" peripheral TCR repertoire (Arstila et al., 1999). In the absence of pathology, the TCR repertoire is fairly stable through time within individual subjects (Malthora et al., 1992). Therefore, it can act as a low-resolution molecular "fingerprint" of the cell-mediated branch of an individual's immune system, and be

used to study the diversity and dynamics of the T-cell compartment. The appearance of the peripheral TCR repertoire is closely associated with the MHC type of the subject. Indeed, in seminal studies, Silver and colleagues have clearly shown that TCR repertoire identity increases linearily with the number of shared MHC alleles (Gulwani-Akolkar et al., 1991). Furthermore, repertoires of monozygotic twins are more closely related than those of haploidentical subjects, underscoring the contribution of minor histocompatibility antigens and/or other as yet unindentified genes to the generation of the mature T-cell pool (Akolkar et al., 1993; Hawes et al., 1993; Malthora et al., 1992). As will be discussed below, this property of the TCR repertoire imposes stringent limitations on the kind of study groups on which these typing experiments can be performed. In particular, cross-sectional studies are notably difficult to interpret unless some measure of human leukocyte antigen (HLA) matching has been put in place, as idiotypic repertoire differences tend to act as confounding factors.

The TCR repertoire can be analyzed by means of a number of molecular biology techniques. Multiparallel semiquantitative polymerase chain reaction (PCR) assays based on complex sets of V-gene-specific oligonucleotide primers were initially introduced (Choi et al., 1989; Genevee et al., 1992; Hall and Finn, 1992; Labrecque et al., 1993; Panzara et al., 1992; Rebai et al., 1994; Soudeyns et al., 1993), soon followed by RNase protection assays (Baccala et al., 1991) and automated run-off-based methods such as the "Spectratyping" (Chen et al., 1995), "Immunoscope" (Pannetier et al., 1993, 1995), and "GeneScan" techniques (Assaf et al., 2000). High-definition repertoire analysis can also be performed using TCR DNA heteroduplex mobility shift assays (HMA), which reveal information relative to the clonality of T-cell subsets expressing specific TCR V-region genes and allow the detection of expanded clonotypes within complex T-cell populations (Shen et al., 1998; Soudeyns et al., 2000; Wack et al., 1996). Due in part to the availability of well-characterized primer sets, PCR-based analysis furnishes a somewhat complete picture of the repertoire. However, the specificity, kinetics and linearity of amplification need to be rigorously controlled to validate experimental protocols (Hall and Finn, 1992). Alternatively, TCR repertoire typing can be performed using flow cytometry and TCR V-region-specific monoclonal antibodies (mAbs). This method presents several notable advantages over PCR-based techniques: a) it is rapid, technically simple, highly reproducible, and results generated can be directly interpreted; b) it is based on cell-surface expression of the TCR and not on levels of TCR mRNA, which are known to fluctuate in concert with the state of T-cell activation (Maguire et al., 1990); and c) multiple markers can be tested at once, allowing the analysis of complex cellular phenotypes and characterization of specific T-cell subpopulations. Close to 100 different TCRBV-specific mAbs have been derived to date, and β chain repertoire coverage currently exceeds 75% with commercially available mAbs alone. A comprehensive listing of the mAbs most commonly used in TCRBV typing can be found in Posnett et al. (1996). TCRAV-specific mAbs are much less common than their anti-TCRBV

counterparts, possibly because TCR α chain V-region determinants are somewhat less immunogenic. In the majority of cases, epitopes recognized by V-specific mAbs have not been precisely mapped. However, the important issues of V-gene specificity, cross-reactivity, and correspondence with ancient and current TCRBV nomenclatures have been addressed in a recent workshop (Posnett et al., 1996). For all these reasons, flow cytometric analysis has become the "gold standard" for studying TCR repertoire variations.

When combined with other techniques of clonal analysis, TCR repertoire typing by flow cytometry can yield a detailed picture of the kinetics, diversity, and persistence of T-cell responses during the course of a pathogenic process. As a prime example, alterations in the TCR β chain repertoire observed in various mice strains were linked to infection with mouse mammary tumor viruses (MMTV) expressing viral superantigens (SAgs), or to the endogenous presence of these MMTVs as germ-line integrants (Dyson et al., 1991; Frankel et al., 1991; Marrack et al., 1991; Woodland et al., 1991). SAgs are thought to form a bridge between MHC class II molecules and the TCR, leading to the activation of large subsets of T cells expressing specific TCRBV families (Lavoie et al., 1999). This is because the TCR β chain and, in particular, the CDR4 segment, were found to be the central determinants of T-cell reactivity to bacterial and viral SAgs (Pullen et al., 1990, 1991; Reinherz et al., 1999). MMTV SAgs were shown to play a critical role in the viral life cycle and in host-pathogen interactions (Golovkina et al., 1992; Held et al., 1996). Since these discoveries, TCR repertoire analysis has been used with varying success as an indirect attempt to reveal the presence of putative SAgs in a number of animal and human pathologies. Among others, these include rheumatoid arthritis (Paliard et al., 1991), multiple sclerosis (Utz et al., 1993), toxic shock syndrome (Scholl et al., 1989), as well as infection with rabies virus (Lafon et al., 1992), Epstein-Barr virus (EBV) (Smith et al., 1993), cytomegalovirus (CMV) (Dobrescu et al., 1995, 1995b), and human immunodeficiency virus type 1 (HIV-1).

TCR REPERTOIRE ANALYSIS IN HIV-ASSOCIATED DISEASE

The Superantigen Hypothesis

Because acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus, a perfectly reasonable parallel was drawn with MMTV infection, according to which the progressive depletion of $CD4^+$ T cells observed in HIV disease could be linked to sequential activation and deletion of $CD4^+$ T-cell subsets by a polymorphic, HIV-associated SAg (Janeway, 1991). It was soon reported that TCRBV-specific deletions of T-cell subsets and various other perturbations of the TCR repertoire could indeed be readily observed in HIV-infected patients (Boldt-Houle et al., 1993; Dalgleish et al., 1992; De Paoli et al., 1993; Hodara et al., 1993; Imberti et al., 1991). Overall, perturbations reported were inconsistent, did not involve preferential deletion of $CD4^+$ T-cell

subsets, and were not correlated with clinical parameters, including the stage of HIV disease. In some cases, similar investigations by other groups even failed to reveal significant repertoire changes (Boyer et al., 1993; Posnett et al., 1993). However, because all of these early cross-sectional studies did not take into consideration the outbred nature of human populations and MHC-type dependence of the repertoire, the significance of the observed variations in TCR β chain expression levels was misappreciated. Indeed, to be able to reveal relevant repertoire differences, such comparisons need to be drawn between subjects who are at least partly haplo-identical (Gulwani-Akolkar et al., 1991). One such approach was to study a cohort of HIV-discordant monozygotic twins, in whom HLA identity was by definition complete. Repertoire analysis revealed significant differences in the levels of expression of multiple TCRBV families, both in $CD4^+$ and $CD8^+$ T cells, and the incidence of these perturbations appeared to increase along with the progression of HIV disease (Rebai et al., 1994). Similar differences were never observed in pairs of uninfected twins, and therefore had to be related to opportunistic infections or HIV infection itself. An alternative approach involved simultaneous testing of several distinct lymphoid subcompartments (i.e., peripheral blood, spleen, and lymph nodes) within the same HIV-infected individual. In two such studies, repertoire typing by PCR or flow cytometry revealed significant differences in the expression levels of several TCRBV subsets (Soudeyns et al., 1993, 1994). Because HIV replicates predominantly within peripheral lymphoid organs during chronic infection (Embretson et al., 1993; Pantaleo et al., 1991, 1993), it was presumed that the origin of the repertoire disparities observed between these lymphoid compartments was related to regional differences in HIV-1 viral load, thus supporting the notion that HIV infection led to qualitative changes in the composition of T-cell subsets.

An independent line of evidence in support of the existence of an HIV-associated SAg was provided by Posnett and colleagues. In accordance with the MMTV model (Held et al., 1993), these investigators hypothesized that TCRBV-specific SAg-mediated activation of T cells would result in increased HIV replication within these cell subsets. Indeed, HIV-1 viral load was found to be significantly greater in $CD4^+$ T cells expressing TCRBV12S1 than in those expressing TCRBV6S7 (Laurence et al., 1992). This subset-specific viral "reservoir" was only observed in presence of CMV coinfection, suggesting the existence of a CMV-associated herpesvirus SAg acting to enhance HIV replication in *trans* (Dobrescu et al., 1995a, b). However, because no preferential expansions of TCRBV12S1 T cells were observed in these subjects, the significance of these observations in the context of HIV pathogenesis remains unclear.

Most of these early reports were, to some degree, consistent with the existence of an HIV-encoded or HIV-associated SAg. Indeed, as the TCR repertoire is remarkably stable through time, TCRBV-specific perturbations are not seen in healthy individuals, or at least not to any comparable extent. However, the apparent randomness in the identity of TCRBV subsets that showed perturbations in HIV infection became increasingly difficult to reconcile with the

effects of a unique HIV-encoded or HIV-associated superantigenic determinant, be it heterogenous or polymorphic. Furthermore, no direct evidence was provided that allowed the identification of a common molecular species, HIV-encoded or otherwise, involved in the induction of these multiple TCR repertoire variations. Taken together, these considerations slowly led to the demise of the "superantigen hypothesis." What then do these repertoire perturbations represent? A large part of the answer came from the study of primary HIV infection.

TCR Repertoire Analysis in Primary HIV Infection

The natural history of HIV-associated disease follows a characteristic pattern. Shortly following infection of the host, most patients experience a febrile illness of varying duration and severity that coincides with transient, high-level HIV-1 viremia and with a sudden decline in $CD4^+$ T-cell counts (Clark et al., 1991; Daar et al., 1991; Tindall et al., 1989). It is during this initial phase that HIV disseminates throughout the peripheral lymphoid organs and that long-term viral reservoirs are established (Chun et al., 1998; Soudeyns and Pantaleo, 1999). Symptoms of primary HIV infection (PI) subside along with the down-regulation of viremia, which results from the appearance of host HIV-specific cell-mediated immune responses (Koup et al., 1994; Musey et al., 1997; Safrit et al., 1994). The disease then enters the so-called chronic phase, during which HIV replication and $CD4^+$ T-cell depletion continue unabated within the peripheral lymphoid system. To determine whether the high levels of antigen routinely detected during PI would influence the T-cell repertoire, investigators performed TCRBV typing in a longitudinal manner in a small cohort of HIV-infected subjects who were identified prior to seroconversion. Although our studies and those of others failed to reveal widespread deletion of specific T-cell subsets (Cossarizza et al., 1995a, b), dramatic perturbations of the TCRBV repertoire were observed, including transient high-level expansions of $CD8^+$ T cells expressing specific TCRBV determinants (Pantaleo et al., 1994). Consistent with ongoing antigen-specific selection, these expanded populations were comprised of one to a few T-cell clones sharing structural similarities in the layout of the TCR β chain CDR3 loop (amino-acid sequence conservation, focusing of CDR3 length, biased J-region usage) (Pantaleo and Fauci, 1995; Pantaleo et al., 1994). Accordingly, these cells bore activation markers (HLA-DR) and exhibited high levels of cytotoxic activity directed against autologous targets expressing HIV-1 antigens, and therefore represented subsets of HIV-specific cytotoxic T lymphocytes (CTL) that had expanded during the course of the primary antiviral cell-mediated immune response (Pantaleo et al, 1994). Since then, transient TCRBV-specific expansions of virus-specific $CD8^+$ T cells have been observed during the acute stage of several other viral diseases, including simian immunodeficiency virus (SIV) infection (Chen et al., 1995), measles (Mongkolsapaya et al., 1999), and infectious mononucleosis (Callan et al., 1996). Perturbations of the $CD8^+$ T-cell repertoire were also shown to occur

throughout the chronic phase of HIV-1 infection (Gorochov et al., 1998; Connors et al., 1997). TCRBV specificity of these T-cell expansions can be explained by the fact that, although CDR3 remains the primary determinant of antigenic specificity, other V-region residues and structures located outside of CDR3 are known to be important for the recognition of Ag-MHC complexes (Casanova et al., 1991; Garboczi et al., 1996; Garcia et al., 1998). Thus, TCRBV restriction can be viewed as a normal result of a generalized T-cell recruitment and expansion process associated with the emergence of primary antiviral cell-mediated immunity.

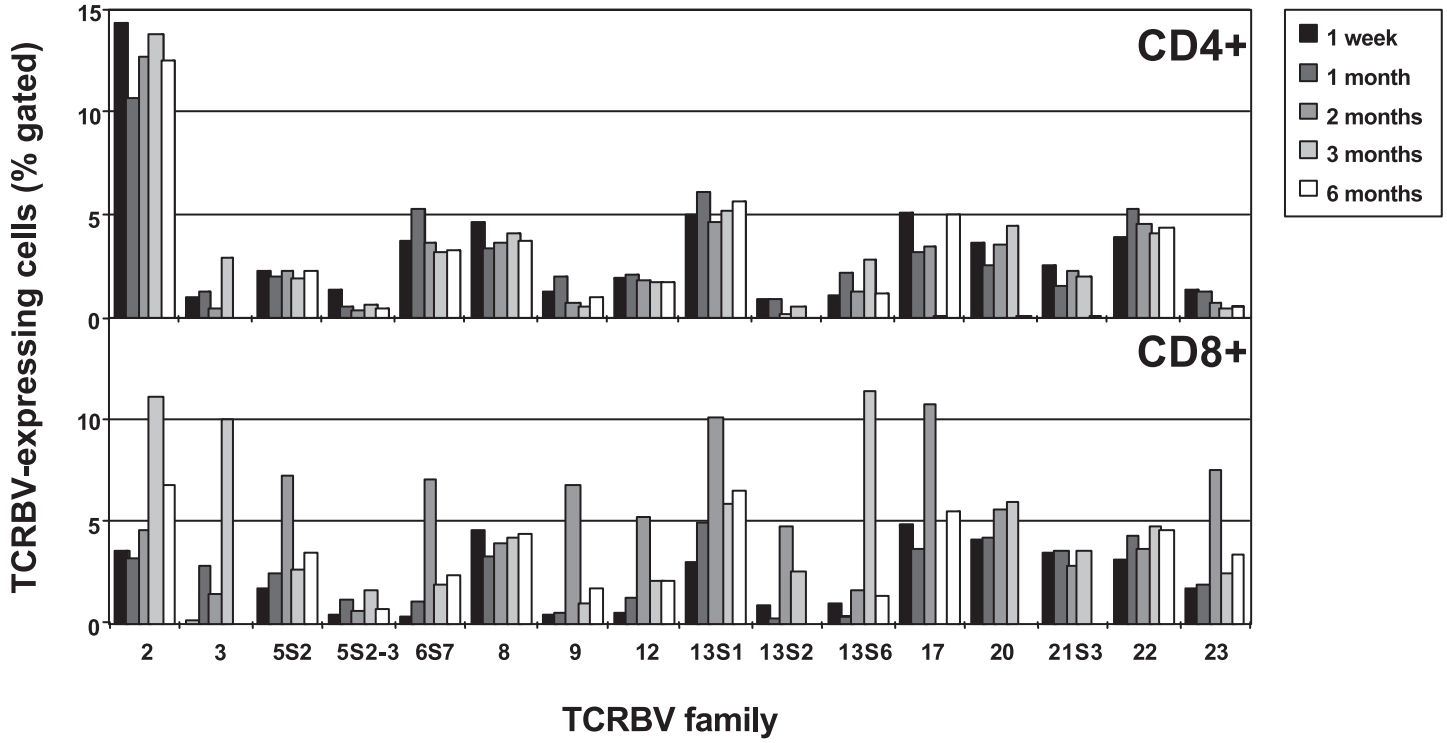
TCR repertoire analysis was also used to examine the global mobilization of the T-cell compartment during PI. By stratifying 30 patients according to the number and magnitude of TCRBV-specific expansions observed during PI, it was shown that the incidence and relative amplitude of these expansions were significantly correlated with the rate of $CD4^+$ T-cell decline (Pantaleo et al., 1997b). In addition, PI patients exhibiting single, high-level expansions of T cells (>10-fold over baseline) were shown to experience unusually rapid HIV disease progression. These results confirmed that immunological and virological events taking place during PI have a strong influence on the long-term prognosis of HIV-associated disease, and underlined the critical role of host factors in shaping HIV pathogenesis (Pantaleo et al., 1997b; Vanhems et al., 2000).

Interestingly, the temporal persistence of HIV-specific CTL clones within patients was shown to be quite variable, with a substantial proportion of HIV-specific CTLs disappearing during PI (Pantaleo et al., 1997a). The rapid kinetics and completeness of this disappearance were consistent with clonal deletion induced by high levels of viral antigens. This phenomenon was initially described in the murine lymphocytic choriomeningitis virus (LCMV) model, used extensively for the study of noncytopathic viral infection (Zinkernagel, 1996). In this system, deletion of antigen-specific CTL expressing high-affinity TCRs was shown to be driven by elevated levels of antigen circulating throughout the lymphoid compartment, and appeared to contribute to the establishment and maintenance of viral persistence (Moskophidis et al., 1993; Oxenius et al., 1998). In HIV-1 infection, important antigen loads are observed throughout the duration of the disease, as viral particles are trapped and accumulate within follicular dendrocyte networks (Embretson et al., 1993; Pantaleo et al., 1993). However, peak circulating antigen levels are reached early on during PI, coincident with the emergence of HIV-specific cell-mediated immune responses. This may very well explain why clonal exhaustion appears to occur preferentially during PI. As discussed previously, the TCR repertoire is very large, not infinite, and only a limited number of peripheral T-cell clones in a given individual can be expected to recognize a specific Ag/MHC combination. In murine systems, this number has been estimated to range between 20 and 200 T-cell clones per MHC class I epitope (Maryanski et al., 1996). Therefore, if numbers of these clones disappear early during infection, this might have a negative impact on the long-term ability of the host to control the replication of

the viral pathogen, and might contribute to the establishment of persistent infection. During PI, expanded HIV-specific CTL clones were not only detected in peripheral blood, but also within lymph node samples taken from the same patients (Pantaleo et al., 1997a). However, the levels of representation of several of these clones was shown to be several orders of magnitude lower in the nodes than in the circulation, both by PCR and by precursor frequency analysis (Pantaleo et al., 1997c). This accumulation took place before the down-regulation of HIV-1 viremia, which is normally associated with the emergence of virus-specific cell-mediated immunity and with the transition between acute and chronic HIV infection. This is quite counterintuitive, inasmuch as $CD8^+$ T cells are known to home in on sites of high-level viral replication so they can mediate their cytotoxic function in situ. However, during PI, HIV replication is focalized within the lymph nodes, resulting in massive localized accumulation of viral antigens. Lymph nodes are also the anatomic site where antigen-specific cell-mediated immune responses are initiated and matured. Thus, it was postulated that the high antigen load routinely detected in lymph nodes during PI resulted in massive stimulation and rapid egress of HIV-specific CTL from that compartment. Although the precise manner by which this is achieved is currently unknown, this mechanism clearly holds the potential to shift the equilibrium existing between viral replication and development of host immune responses in favor of the former.

Pediatric HIV Infection

Vertical transmission represents the most common route via which children and infants become infected with HIV-1. In many respects, vertical HIV transmission represents a form of PI, one in which the time of infection and the source of the infecting virus can be clearly identified. Accordingly, our group and others have observed transient TCRBV-specific expansions of $CD8^+$ T cells in HIV-infected children and infants (Halapi et al., 1996; Silvestri et al., 1996; Soudeyns et al., 1993) (Fig. 7.1). As in PI, detailed analysis of the CDR3 layout revealed a surprisingly important degree of structural conservation, including CDR3 size restriction and biased beta chain junctional region usage, both of which are fully consistent with antigen-specific selection processes (McHeyzer-Williams and Davis, 1995; Rock et al., 1994). In fact, a large part of these $CD8^+$ T-cell expansions were comprised of consecutive waves of amplified T-cell clones exhibiting differential persistence, reflecting the complex dynamics of the T-cell mobilization process associated with cell-mediated immune responses (Silvestri et al., 1996; Soudeyns et al., 2000b). Expansions of $CD8^+$ T cells were also observed in uninfected children born to HIV-infected mothers, albeit at a significantly lower frequency (Soudeyns et al., 2000a). In accordance with these results, children born to HIV-infected mothers are known to mount potent HIV-specific CTL responses that are thought to play an important role in the prevention of vertical HIV transmission (Cheynier et al., 1992; Rowland-Jones et al., 1993). Most interestingly, transient expansions of $CD4^+$ T cells, which


CD4+
CD8+
1 week
1 month
2 months
3 months
6 months
TCRBV-expressing cells (% gated)
15
10
5
0
2
3
5S2
5S2-3
6S7
8
9
12
13S1
13S2
13S6
17
20
21S3
22
23
TCRBV family

are rarely seen in adult PI, were also observed in these children, although not as frequently as those involving $CD8^+$ cells (Soudeyns et al., 2000b) (Fig. 7.1). The functional significance of these $CD4^+$ T-cell expansions has not been elucidated. However, in stark contrast with $CD8^+$ expansions, these cells exhibited a high level of polyclonality and an almost total lack of conservation in the CDR3 layout. Because $CD4^+$ T-cell responses are markedly more polyclonal than those involving $CD8^+$ T cells (Maini et al., 1999), these observations are nevertheless consistent with the possible involvement of these expanded subsets in ongoing antigen-specific T-helper responses (Soudeyns et al., 2000a). Alternatively, the apparent polyclonality might simply reflect continual HIV-mediated depletion and remodelling of the $CD4^+$ T-cell compartment.

Effects of Antiretroviral Therapy

The introduction of highly active antiretroviral therapy (HAART) has been credited with the recently recorded declines in the incidence of HIV-related morbidity and mortality (Palella et al., 1998). HAART been shown to promote a variable but significant recovery of immune responsiveness in treated patients (Autran et al., 1997; Fleury et al., 1998, and references therein). Paradoxically, the intensity of the CTL response and the frequency of HIV-specific Th and CTLs have been shown to decline following prolonged periods of viral suppression (Gray et al., 1999; Ogg et al., 1999; Pitcher et al., 1999). This was presumed to represent an indirect result of the effective suppression of viral replication and concomitant reduction in the amounts of circulating HIV antigens available to prime and maintain high-level immune responses. At the level of the TCR repertoire, this effect is translated by a significant decline in the frequency and magnitude of perturbations in the $CD8^+$ T-cell subset (Connors et al., 1997; Gorochov et al., 1998). In contrast, the rate of normalization of the $CD4^+$ T-cell repertoire is variable following introduction of therapy, reflecting

Figure 7.1. Longitudinal analysis of the TCR β chain repertoire in $CD4^+$ and $CD8^+$ T cells from a HIV-negative infant born to an HIV-infected mother. The child was followed from birth until 6 months of age. Vertical bars correspond to the relative levels of representation of specific TCRBV families in samples of peripheral blood mononuclear cells (PBMC). Direct staining of PBMCs was performed using fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (mAbs) specific for TCRBV2S1 (E2.2E7.2), TCRBV3S1 (LE89), TCRBV5S2 (36213), TCRBV8 (56C5), TCRBV13S1 (IMMU222), TCRBV13S6 (JU74.3), TCRBV17 (E17.5F3.15.13), TCRBV20S1 (ELL1.4), TCRBV21S3 (IG125), and TCRBV22 (IMMU546), obtained from Immunotech (Marseille, France). FITC-labeled anti-TCRBV6S7 (OT145) and anti-TCRBV12 (S511) were obtained from Endogen (Woburn, MA). Unlabeled mAbs used in indirect staining included anti-TCRBV5S2-3 (MH3-2), anti-TCRBV9 (MKB1), anti-TCRBV13S2 (13.2), and anti-TCRBV23S1 (HUT78). In these cases, FITC-conjugated goat anti-mouse IgG (Life Technologies, Burlington, Ontario Canada) was used as a secondary antibody. Cells were counterstained with phycoerythrin (PE)-conjugated anti-CD4 (*top panel*) or anti-CD8 mAbs (*bottom panel*) (Becton-Dickinson, Mountain View, CA). Analysis was performed on 10,000 events, gated according to forward and side-scatter, on a FACScan driven by the CellQuest software package (Becton-Dickinson). Results are expressed as percentage of cells in the lymphocyte gate.

individual differences in the rate of regeneration and redistribution of $CD4^+$ T cells from the central and peripheral lymphocyte pools (Douek et al., 1998; Pakker et al., 1998a, b). Clinical guidelines currently recommended that subjects diagnosed with PI immediately initiate an aggressive regimen of HAART, using one of several effective combinations of HIV-1 reverse transcriptase and protease inhibitors (Ho, 1995). The rationale behind HAART treatment of PI is three-fold: a) to rapidly suppress viral replication and limit the spreading of HIV to the peripheral lymphoid compartments; b) to alleviate the symptoms associated with PI and restore $CD4^+$ T-cell counts; and c) to shield initial HIV-specific cell-mediated immune responses against HIV-induced cytopathology and/or functional impairment—it was suggested that these responses might otherwise become irreversibly compromised during and after PI (Rosenberg et al., 1997). In this context, TCRBV repertoire analysis revealed that introduction of HAART during PI influenced the mobilization of T-cell subsets by a) inducing a more rapid TCRBV repertoire stabilization than that seen in absence of treatment; b) reducing the global level of T-cell oligoclonality; and c) initiating a biphasic decline in the peripheral representation of HIV-specific $CD8^+$ CTL clones (Soudeyns et al., 2000a). It has been hypothesized that such progressive reduction in the frequency of HIV-specific CTL might eventually lead to a loss of control over residual levels of HIV replication, but there has been no evidence so far that this is in fact the case. However, given the presumed importance of residual replication with respect to the emergence of drug-resistant variants, the use of immunomodulators, therapeutic vaccines, and "structured therapy interruptions" has been advocated (Pantaleo, 1997; Rosenberg et al., 2000).

CONCLUSION

As a result of the somatic gene rearrangement process, each T-cell clone expresses a TCR endowed with uniquely specific antigenic recognition properties. The sum of all TCRs expressed by the host is termed the TCR repertoire, and it can be analyzed by flow cytometry. HIV-1 infection is characterized by an unrelenting depletion of $CD4^+$ T-helper cells, leading to the progressive appearance of a lethal state of immunosuppression. The presence of TCR β chain repertoire perturbations was revealed in various groups of HIV-infected subjects, including HIV-discordant monozygotic twins, patients undergoing primary HIV infection, and children born to HIV-infected mothers. Although these studies did not lead to the identification of an HIV-associated SAg, TCR repertoire analysis was instrumental in the discovery of several fundamental characteristics of primary antiviral cell-mediated immune responses, including a) oligoclonal expansion and deletion of antigen-specific CTLs; b) transient polyclonal expansions of $CD4^+$ T cells; and c) differential compartmentalization of HIV-specific CTL. This led to advances in the understanding of the dynamics of cell-mediated immune responses during acute and persistent viral

infections, and contributed to the recognition of host factors as key determinants of HIV disease progression.

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8

Gamma-Delta ($\gamma\delta$) T Cells and HIV-1 Infection

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GENERAL OVERVIEW OF γδ T-CELL BIOLOGY

This review on γδ T cells in human immunodeficiency virus 1 (HIV-1) infection will focus, unless otherwise indicated, primarily on human T cells. It is aimed at providing the tools to understand the role of γδ T cells in HIV infection.

αβ T cells, B cells, and γδ T cells constitute the three lymphocyte lineages present in all jawed vertebrate species. Among $CD3^+$ T cells, γδ T cells were the most recently described lymphocyte subset. Since the original description of the T-cell receptor (TCR) γ chain in 1984 and the isolation of a second heterodimeric TCR in 1986, extensive studies have been performed on the role and function of γδ TCR-bearing cells in human immune responses (Bank et al., 1986; Born et al., 1987; Brenner et al., 1986; Chien et al., 1987; Pardoll et al., 1987; Saito et al., 1984; Weiss et al., 1986). Considerable progress has been made during the last decade, resulting in significant understanding of many biological and functional features of this T-cell subset. However, a large number of issues in the γδ T-cell field remain unresolved, such as the manner in which antigens are processed and presented to γδ TCRs.

It has been proposed that γδ TCR genes are phylogenetically more primitive than the αβ TCR genes (Herzenberg, 1989; Richards and Nelson, 2000). However, other authors propose that there is no substantial evidence to sup-

port a more ancient origin for γδ TCR genes (Born et al., 1999; Hayday, 2000). γδ TCR genes are present in all animal species, from cartilaginous fish (*Raja eglanteria*, or skate) to mammals (Born et al., 1999; Rast et al., 1997). Although γδ T cells are broadly distributed in all jawed vertebrate species, there are significant interspecies differences in the genes that encode δ and in particular γ chains as well as in the anatomical distribution and abundance of γδ T cells (Anderson et al., 1995; Hayday et al., 1985; Lefranc and Rabbitts, 1989; Lefranc et al., 1989; Six et al., 1996). For example, although γδ T cells represent only 1–5% of peripheral $CD3^+$ cells in primates and rodents, they are abundant in the avian immune system and they account for up to 70% of circulating $CD3^+$ T cells in young ruminants, decreasing to 5–25% with aging (Cooper et al., 1989; Hein and Mackay, 1991). αβ and γδ T cells both use CD3 as an activation coreceptor and both produce cytokines and express cytotoxic effector function (cytotoxic T lymphocytes, or CTL) upon TCR engagement. However, αβ and γδ T cells differ in many important ways. First, in terms of tissue distribution, the majority of $CD3^+$ T cells in the peripheral blood and peripheral lymphoid tissues in humans and mice express the αβ TCR and only 1–5% express the γδ TCR. In contrast, the majority of γδ TCR-bearing T cells are found in epithelial tissues, such as the gut in humans (intestinal intraepithelial lymphocytes, or IELs), and the skin and reproductive tract of mice (Deusch et al., 1991; Goodman and Lefrancois, 1988; Itohara et al., 1990; Stingl et al., 1987).

Second, γδ TCR show a more restricted diversity at the genetic level than αβ TCR. The number of Vγ and Vδ genes in the germ line, and therefore their combinatorial diversity, is limited compared with the number of Vα and Vβ genes. The use of genetic mechanisms, such as N-region diversification among others, increases the diversity of the γδ TCR repertoire (Brenner et al., 1988; Kabelitz, 1992; Lefranc and Rabbitts, 1989; LeFranc et al., 1986; Quertermous et al., 1986a, b). Although the immune system can generate a broad and diverse γδ TCR repertoire, one of the most remarkable characteristics of the γδ T-cell population is the preferential usage of certain V region genes to form the TCR. This bias toward the expression of certain V gene products in part defines the tissue-specific restriction of certain γδ TCRs. For example, two single variable region genes (*TCRGV2S1* and *TCRDV102S1*) encode the Vγ9/Vδ2 chains found in >80% of peripheral blood γδ T cells in adults (Borst et al., 1989; Bottino et al., 1988; Casorati et al., 1989; Kabelitz et al., 1991; Parker et al., 1990; Triebel et al., 1988a, b). In contrast, Vδ1 is preferentially expressed in human spleen and thymus, whereas human IELs carry both Vδ1 and Vδ3 elements (De Libero et al., 1993; Peyrat et al., 1995; Porcelli et al., 1991). The existence of canonical or homogeneous (identical V(D)J composition and junctional sequences) TCRs has been described in mice and humans, adding to the restriction of the γδ repertoire (Davodeau et al., 1993; McVay and Carding, 1999; Tamura et al., 1990).

Third, αβ and γδ T cells differ in the manner in which they recognize antigens. Whereas αβ T cells mainly recognize peptides presented by major histo-

compatibility complex (MHC) molecules, the range of antigens recognized by γδ T cells remains poorly defined, but includes nonpeptidic compounds such as small phosphorylated molecules (Constant et al., 1994; Tanaka et al., 1994). The recognition of peptide or nonpeptidic moieties seems to be non-MHC restricted and may not require antigen processing or presentation (Lang et al., 1995; Morita et al., 1995).

Fourth, γδ T cells differ from αβ T cells in the expression of the MHC receptors CD4 and CD8. Most γδ T cells in peripheral blood or tissues express neither CD4 nor CD8 (Groh et al., 1989; Lanier et al., 1986; Scott et al., 1990). A small proportion of peripheral blood γδ T cells expresses CD8, and CD4 expression is rare (Moretta et al., 1988). Functional capabilities of γδ T cells are similar to αβ T cells and include cytotoxicity and cytokine secretion. However, γδ T cells may have unique functions such as the regulation of epithelial growth and immunoregulatory functions. γδ T cells may have a role in regulation of antibody production and isotype switching (Hacker et al., 1995; Horner et al., 1995; Munk et al., 1994, 1995; Rajagopalan et al., 1992). γδ T cells were also implicated in regulating αβ T-cell development and modulating αβ T-cell effector function. In vitro experiments with transgenic mice in which TCR γ gene expression was modified and studies with anti-pan-TCR-δ monoclonal antibodies in mice showed altered thymic development and increased peripheral αβ T-cell responses, such as higher proliferation and interleukin (IL)-2 production (Ferrick et al., 1989a, b, c, 1990, 1991; Kaufmann et al., 1993). In vivo depletion of γδ T cells has been shown to alter T-cell-dependent hypersensitivity, tolerance, and host resistance to pathogens, suggesting a role for γδ in regulation of αβ TCR (Vincent et al., 1996). This regulatory mechanism may be mediated by Fas/Fas ligand (Fas-FasL) interaction and apoptosis and may be important to restrict responses of αβ T cells and other Fas^+ effector cells (Born et al., 1999). In addition, γδ T cells have been implicated in regulation of early and late stages of inflammation in different diseases such as influenza, listeriosis, and mycobacterial infections (Carding, 1990; Carding et al., 1990a; D'Souza et al., 1997; Fu et al., 1994; Hsieh et al., 1996). Furthermore, a role in regulation of tolerance to ingested or inhaled antigens and tissue grafts has also been described for γδ T cells (Fujihashi et al., 1990, 1997; McMenamin et al., 1991, 1994; Wildner et al., 1996; Vaessen et al., 1996; Vandekerckhove et al., 1990). A role for γδ T cells has been described in numerous human diseases, including bacterial, protozoal, and viral infections, tumor immunity, and autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus.

THE γδ TCR AND THE γδ GENE FAMILIES

Human $CD3^+$ T cells express one of two TCR heterodimers: either an α chain with a β chain ($\alpha\beta TCR^+$ T cells) or a γ chain combined with a δ chain ($\gamma\delta TCR^+$ T cells). The γ and δ chains consist of one variable region and one

constant domain (Haas et al., 1993; Raulet, 1989). The structure of human γδ TCR resembles that of the αβ TCR in that they organize each V and C domain into immunoglobulin (Ig)-like folds linked by intradomain disulfide bonding (Li et al., 1998). The V domains are subdivided into framework and hypervariable regions with surface-exposed complementary determining regions (CDRs) (Li et al., 1998). The structure of the γδ TCR is much closer to that of the αβ TCR than to the structure of the Ig receptor. Although several structural features are common to both types of TCR, γδ TCR have distinctive properties. The γδ TCR has charged amino acid substitutions in particular positions of the Vγ and Vδ chains and uses corresponding alignments in CDR1 and CDR2 of different subfamilies of Vγ and Vδ chains. These special alignments may determine the restricted γ/δ chain pairing characteristic of γδ TCRs (Arden et al., 1995a,b). In addition, γδ TCRs show a higher divergence in the CDR1 and CDR2 regions than αβ TCRs and this may expand the diversity of the repertoire of γδ TCR. αβ TCRs are constrained in the CDR1 and CDR2 by the requirement for the binding to MHC molecules (Chien and Davis, 1993; Garcia et al., 1996). Perhaps the most interesting difference between αβ and γδ TCR structure is the diversification of the CDR3 region length and sequence variability exhibited by γδ TCRs (Davis and Bjorkman, 1988; Rock et al., 1994). CDR3 loops are of particular interest because they represent the site of peptide/antigen contact and recognition (Davis and Bjorkman, 1988; Engel and Hedrick, 1988). CDR3 of human TCRδ are 8–21 amino acids long, whereas CDR3 of TCRγ are shorter (1–12 amino acids) but also variable in size (Rock et al., 1994). In contrast, CDR3 loops of TCR α and β chains are typically similar in length (6–12 amino acids), reflecting the size constraints imposed by peptide antigens bound to the MHC groove. The length variation in CDR3 γ/δ may reflect greater heterogeneity in the size of γδ T-cell ligands. In this sense, antigen recognition by γδ TCR may be more similar to Ig recognition of antigen than to that of αβ TCR recognition of the MHC-peptide complex (Chien et al., 1996). The crystal structure of the human TCR Vδ (Vδ3-Dδ2-Jδ1) chain suggests that its overall structure shares features of both αβ TCR and Ig receptors, both in the framework and CDR regions (Li et al., 1998). It has been proposed that these structural features may function to couple Ig-like recognition properties, such as recognition of native antigens, with cellular effector functions, such as target cell lysis or interferon (IFN)-γ secretion (Sciammas et al., 1994).

Each chain of the γδ TCR is encoded in different loci. The human γ locus (*TCRG*) is located on chromosome 7 and consists of a series of V genes (Vγ) located 5′ of two constant genes (*TRGC* = Cγ 1 and Cγ 2), each one preceded by J segments (*TRGJ*), three upstream of Cγ 1 and two located upstream of Cγ 2 (Bensmana et al., 1991). The use of Cγ1 or Cγ2 gene segment determines whether the γδ heterodimer is disulfide linked or not (type 1 vs. type 2 respectively) (Lefranc and Rabbitts, 1989). There are no Dγ segments. The Vγ region comprises 14 genes (*TRGV* = six pseudogenes, eight functional genes). These *TRGV* genes belong to four subfamilies (I–IV) whose members share amino acid sequence homology. The Vγ I subfamily consists of nine members, five

functional genes and four pseudogenes; the other three subfamilies contain only one member each and there are two pseudogenes that do not belong to any of these subfamilies. Different nomenclature has been proposed for designating human TCR genes. The WHO-IUIS Nomenclature Subcommittee on TCR Designation (WHO-IUIS, 1995) and the International ImMunoGeneTics database (IMGT, URL available at *http://imgt.cnusc.fr:8104*) is the one currently used the most (Arden et al., 1995a; Lefranc et al., 1998, 1999).

The human TCRδ (*TCRD*) genes are located on chromosome 14 within the TCRα (*TCRA*) locus, between the Vα and the Jα gene segments (Griesser et al., 1988; Satyanarayana et al., 1988). The Vδ genes are actually interspersed with Vα gene segments (Loh et al., 1988; McVay and Carding, 1999; Miossec et al., 1991; Takihara et al., 1989a, b). Eight human Vδ genes (*TRDV*) have been described. Vδ1 and Vδ2 genes are located 5′ of Dδ segments and Vδ3 is downstream of Cδ in an inverted transcriptional orientation (Hata et al., 1989; Takihara et al., 1989a). Vδ4 to Vδ8 are members of the Vα gene families and can rearrange either with Jα and Cα or Jδ and Cδ segments to form TCR-α or TCR-δ chains, respectively. The TCR-δ locus is completed with three Dδ (*TRDD* = Dδ1–3) segments, four Jδ (*TRDJ* = Jδ1–4) segments and a single Cδ segment (*TRDC*) (Davodeau et al., 1994; Loh et al., 1988; Takihara et al., 1988).

The limited number of germ-line V genes and D and J segments is the cause of the low combinatorial diversity of the γδ TCRs compared with the αβ TCRs (Davis and Bjorkman, 1988). The γδ TCR repertoire is even more restricted by preferential V gene usage. However, it has been estimated that the potential number of different γδ TCRs in mice actually exceeds (10^{20}) that of αβ TCRs (10^{15}) or B-cell Ig receptors (10^{11}) (McVay and Carding, 1999). The main mechanisms that account for the diversification of γδ TCRs include: a) diversification of the N region (nucleotide insertion and modification of TRDV-(D)-J junctional sequences); b) translation of the D region in three different reading frames in the δ chain; and c) use of multiple contiguous D segments to form the δ chain (Kabelitz, 1992; LeFranc et al., 1986; Sturm et al., 1989). Consequently, the antigen repertoire of γδ T cells is potentially greater than that for αβ T cells. To what extent this diversity is used by γδ TCRs is not known.

ORIGIN AND DISTRIBUTION OF γδ T CELLS

Ontogeny of Human γδ T Cells

Prenatal development of γδ T cells in humans can be divided into two phases: prethymic phase (before thymic development by the 8th week of gestation) and intrathymic phase (after thymic development, 8th week). γδ T cells are generated before the 8th week of gestation in two sites: fetal liver and primitive intestine (McVay and Carding, 1996; McVay et al., 1998; Wucherpfennig et al., 1993). Productive gene rearrangements and transcripts of both Vγ and Vδ

chains were detected as early as in the 5th week. During the first 12 weeks of gestation, γδ T cells are the only T cells detected in fetal liver. These primitive γδ T cells express predominantly Vγ9/Vδ2 TCR and the Vγ9 chain shows limited structural diversity (McVay and Carding, 1996; Carding et al., 1990b). Interestingly, these canonical receptors are functional and show the same antigen specificity as adult Vγ9/Vδ2 T cells, i.e., they respond to mycobacteria by proliferating and with cytokine secretion (Davodeau et al., 1993). On the other hand, productive rearrangements and expression of both Vδ and Vγ chains have been described in fetal gut by the 6th week (McVay et al., 1998; Spencer et al., 1986). By week 8, the Vδ chain repertoire differs between liver and gut, with Vδ1 being the dominant transcript in the intestine (Holtmeier et al., 1997). The Vδ1 transcripts generated by the 6th to 8th week in gut have extensive junctional diversity (McVay et al., 1998). The origin of these primitive γδ T cells detected in the gut is not clear, and three possibilities have been proposed: a) they are generated in situ (McVay et al., 1998); b) they traffic from the liver (McVay and Carding, 1999); and c) they are maternally derived (Mincheva-Nilsson et al., 1997; White et al., 1994).

Fetal liver-derived progenitors seed the thymic rudiment by the 8th week of gestation (Haynes and Heinly, 1995; Lobach and Haynes, 1987). The exact role of the thymus in γδ T-cell development is not clear. Some studies suggest that some fetal-derived γδ TCR-bearing cells could migrate and seed the thymus (McVay et al., 1998). The thymus then would be a place to mature γδ T cells with already rearranged TCR genes. Changes in the profile of Vδ gene expression during thymic development and the finding of *TCRD* and *TCRG* genes in germ-line configuration suggest that thymus also can have a role in the generation, differentiation, and thymic selection of progenitor cells (Blom et al., 1998; Krangel et al., 1990; McVay et al., 1991a, b).

Thus, γδ T cells seem to be originating from progenitor cells in both extrathymic sites and in the thymus during fetal development.

γδ T-Cell Repertoire Changes in Postnatal Life and Varies in Different Tissues

In adults, γδ T cells represent about 5% of $CD3^+$ cells in peripheral blood. Between 50% and 98% of the circulating γδ T cells express Vδ2 (*TRDV2-DJ1-DC*) paired with Vγ9 (*TRGV9-GJP-GC1*) and a minority (3–9%) have TCRs with either Vδ1 or Vδ3 elements (Jitsukawa et al., 1987; Lanier et al., 1988; Moretta et al., 1988; Peyrat et al., 1995; Triebel and Hercend, 1989). This bias toward the expression of Vγ9Vδ2 T cells is not observed in cord blood, where the diversity of γδ TCRs is broader (Morita et al., 1994; Parker et al., 1990). The γδ T cells represent approximately 2% of $CD3^+$ cells in umbilical cord blood, with 50% expressing Vδ1 and 25% carrying Vδ2. Interestingly, Vδ1 cells remain predominant in thymus at all ages (65% Vδ1, 17% Vδ2) whereas Vδ1 expression in peripheral blood decreases from 50% at birth to $<10\%$ by the ninth decade of life. Vδ2 expression starts to increase during the first decade of

life and reaches a mean of 75% in adults (Parker et al., 1990). Apart from changes in Vγ and Vδ expression, there is a tendency to develop a more oligoclonal γδ T-cell repertoire with age, which suggests an antigen-mediated selection of γδ TCRs (Giachino et al., 1994).

Sites of preferential γδ T-cell location in postnatal life include thymus, intestinal epithelium, and red pulp of the spleen (1–17% of T cells), where γδ T cells predominantly express Vδ1 paired with Vγ other than Vγ9 (Bordessoule et al., 1990; Casorati et al., 1989; Deusch et al., 1991; Porcelli et al., 1991; Ullrich et al., 1990). Among IELs, γδ T cells represent between 30% and 40% of all lymphocytes. About 70% of γδ TCR^+ IELs express Vδ1 and 17% Vδ3 (Deusch et al., 1991; Spencer et al., 1989; Ullrich et al., 1991). As in peripheral blood, intestinal γδ T cells tend toward increased oligoclonality with age (Holtmeier et al., 1995; Van Kerckhove et al., 1992). This has been attributed to bacterial antigen-, environmental antigen-, or neuroendocrine hormone-driven selection (Holtmeier et al., 1995).

One unresolved issue in postnatal γδ T-cell development in humans is the extrathymic generation of γδ T cells in the intestine. Generation of γδ T cells in the intestinal cryptopatches has been demonstrated in mice but it is still unproven in humans (McVay and Carding, 1999).

γδ T PHENOTYPE

γδ T-Cell Morphology and Surface Markers

In general, circulating γδ T cells are morphologically similar to αβ T cells. In contrast, Vδ1 expressing T cells in mucosal tissues can adhere to plastic and emit long filopodia when maintained in culture (Ferrini et al., 1989; Grossi et al., 1989).

Most γδ T cells do not express CD4 or CD8 coreceptors (Lanier et al., 1986). A small fraction of γδ T cells express CD8 (20–30%, mainly Vδ1). Expression of CD4 is rare and correlates with little or no cytotoxic activity (Groh et al., 1989; Morita et al., 1991; Scott et al., 1990). The low frequency of expression of CD4 and CD8 coreceptors in most γδ T cells is consistent with the lack of evidence for MHC restriction in antigen recognition.

Other receptors commonly expressed on the surface of αβ and γδ T cells include CD2, CD5, and CD7 (Faure et al., 1988; Groh et al., 1989; Kabelitz and Conradt, 1988). CD28, the ligand for B7 (CD80/86), is absent from the majority of γδ T cells (Groh et al., 1989; Poggi et al., 1987). As CD28 is the main costimulator of αβ T cells, other molecules must be responsible for costimulation of γδ T cells. Candidates include CD40L and vitronectin. A significant percentage of Vγ9/Vδ2 T cells expresses the memory marker CD45RO (Braakman et al., 1991; Hayward et al., 1989; Miyawaki et al., 1990). These γδ T cells also constitutively express perforin and serine esterases suggesting in vivo preactivation (Koizumi et al., 1991; Nakata et al., 1990).

The γδ T cells express surface receptors common on natural killer cells, the so-called natural killer cell receptors (NKRs). There are two kinds of NKRs, inhibitory receptors, or KIR, and activating receptors, or KARs. After binding a particular self-human leukocyte antigen (HLA) class I allele, KIRs transmit inhibitory signals to killer cells (Moretta et al., 1996). In contrast, KARs recognize foreign class I alleles and activate cytolytic activity by killer cells (Lanier et al., 1997). NKRs are expressed by the majority of Vγ9Vδ2 T cells but not on the Vδ1 subset. These receptors may play a role in regulation of γδ T-cell responses to bacterial phosphoantigens and yet unidentified tumor antigens by shifting the activation threshold to high antigenic doses (Carena et al., 1997; Fisch et al., 1997; Halary et al., 1997). Some KIR identified on cytotoxic γδ T cells were found to be important regulators of lysis of Daudi lymphoma cells or virus-infected cells (Moris et al., 1999; Poccia et al., 1997). The KIR found in γδ T cells are p58.1 (KIR2DL1), p58.2 (KIR2DL2), p70 (KIR3DL1), p140 (KIR3DL2), and CD94/NKG2A or B heterodimer (Aramburu et al., 1990; Battistini et al., 1997; Boullier et al., 1998). The KARs are not as well defined and may serve to reinforce the activity of other signaling receptors, such as TCR and KIRs (De Libero, 1999).

γδ T-Cell Effector Molecules And Function

The γδ T cells share functional capabilities with αβ T cells, i.e., cytotoxicity and cytokine production (Haas et al., 1993; Kabelitz, 1999). γδ T cells exert their cytotoxic activity on infected cells or tumor cells (Borst et al., 1987; Bukowski et al., 1994). A variety of infectious agents can elicit cytotoxicity by human γδ T cells: bacteria (*Mycobacterium tuberculosis*, *Yersinia enterocolitica*) (Munk et al., 1990; Tsukaguchi et al., 1995; Young et al., 1997), parasites (*Plasmodium falciparum*, *Toxoplasma gondii*) (De Paoli et al., 1992; Troye-Blomberg et al., 1999) and viruses (HIV, herpes simplex, and Epstein-Barr virus) (Bukowski et al., 1995; Maccario et al., 1993; Wallace et al., 1996). This cytotoxicity is non-HLA restricted but involves specific interactions between γδ TCR and target cells (Dieli et al., 2000; Lang et al., 1995; Morita et al., 1995). The cytotoxic activity is mediated by two major mechanisms: granule exocytosis mediated by granzyme/perforin and/or apoptosis triggered by the Fas/FasL interaction. Preferential use of one of these two pathways has been reported in different diseases (Boullier et al., 1997; Dieli et al., 2000; Troye-Blomberg et al., 1999; Vincent et al., 1996). γδ T cells, similar to αβ T cells, also are a source of cytokines and differential production of IL-2, IFN-γ or IL-4, IL-5, IL-6, and IL-10 has been described for mouse γδ T cells (Ferrick et al., 1995; Fowell et al., 1997; Wen et al., 1998). However, in humans the dichotomy between T-helper (Th)1 and Th2 is less clear than in mice and human γδ T cells are biased to the Th1 phenotype, being very effective producers of IFN-γ (Tsukaguchi et al., 1995). Some stimuli such as phosphoantigens result in secretion of TNF-α (Garcia et al., 1997; Lang et al., 1995). Some IL-2 secretion by γδ T cells has been reported, but it is not sufficient to sustain the γδ T-cell proliferation in culture (Pechhold et al., 1994; Wesch et al., 1997).

Another function attributed to γδ T cells is maintenance of epithelial integrity. This is a singular capability of γδ T cells, which is not observed in αβ T cells. γδ IELs obtained from murine intestinal epithelium secrete growth factors, such as keratinocyte growth factor (KGF) and epidermal growth factor that promote growth of epithelial cells and may mediate tissue repair (Boismenu and Havran, 1994, 1997; King et al., 1999). Further research will be necessary to assess the role of human γδ IELs in epithelial homeostasis.

The fact that γδ T cells can respond rapidly to ubiquitous antigens and produce a wide variety of mediators that affect cells from both the innate and adaptive immune system has suggested that γδ T cells serve as immunoregulatory cells (Born et al., 1999). For example, γδ T cells have been implicated in B cells help and Ig isotype switching in *Paracoccidioides brasiliensis* infection and in lupus nephritis (Horner et al., 1995; Munk et al., 1995; Rajagopalan et al., 1990).

ANTIGEN REPERTOIRE AND RECOGNITION

One of the most important advances in the field of γδ research over the past decade was the discovery that nonpeptidic phosphorylated compounds are antigens recognized by human peripheral γδ T cells. This finding challenged the paradigms of antigen recognition by T cells and opened a novel insight into the biology of the immune system.

Notwithstanding the conservation of γδ TCR^+ T cells among jawed vertebrate species, the antigen repertoire diverges between different species. For example, the characteristic reactivity to phosphoantigens observed in human Vγ9Vδ2 circulating T cells is not seen in mice. On the other hand, empty class II MHC, class Ib MHC, CD48 (Ig gene superfamily), heat shock proteins, and Herpes simplex virus glycoprotein I (gI) are antigens commonly recognized by mouse γδ T cells.

The antigen repertoire for human γδ T cells differs for each γδ T-cell subset. Peripheral blood Vγ9Vδ2 T cells recognize nonpeptidic phosphorylated compounds and reactivity to alkylamines was also recently described for this subset (Bukowski et al., 1999). Vδ1 IELs react against MHC class I-related molecules, MICA and MICB. Some infrequent reactivities have been described in humans which includes heat shock proteins (HSPs) and idiotypic determinants of Igs. More recently, self-recognition of non-polymorphic CD1c molecules by the major tissue γδ T-cell subset Vδ1 has been reported (Spada et al., 2000).

Recognition of Phosphoantigens by Human $V\gamma9V\delta2^+$ T cells

The most common γδ T-cell subset in humans, the Vγ9Vδ2 T cells, can be stimulated by a variety of pathogenic microorganisms. These include intracellular protozoa (*Plasmodium falciparum*, *Leishmania brasiliensis*, *Toxoplasma gondii*), Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogens*), Gram-negative bacteria (*Escherichia coli*, *Salmonella*

typhimurinum, *Yersinia enterocolitica*), and intracellular bacteria (*Mycobacterium tuberculosis*, *Mycobacterium leprae*) (Fournie and Bonneville, 1996). Soon after the observation was made that Vγ9Vδ2 T cells have broad reactivity to infectious agents, the search for microbial antigens was begun. The first candidate antigens were the heat shock proteins, because the HSP 65 was determined to be a potential major stimulus for murine γδ T cells (Born et al., 1990). However, it was soon evident that peptidic antigens were not the most common stimulatory molecules for human γδ T cells and γδ antigenic specificities strikingly diverged from conventional αβ T-cell antigens. The original observation that γδ stimulatory antigens were of low molecular mass and protease resistant came from studies of γδ T-cell responses to mycobacterial antigens (Pfeffer et al., 1990, 1992). Two approaches were undertaken to characterize these molecules, purification from mycobacterial sources and assays with synthetic compounds. In 1994, two groups separately reported the chemical features of the nonpeptidic γδ stimulatory antigens (Constant et al., 1994; Tanaka et al., 1994). The common feature of these compounds was the presence of phosphate groups. These nonpeptidic compounds with phosphoester structures that stimulate γδ T cells are collectively called "phosphoantigens." Subsequent studies by these two groups suggested that there are two classes of phosphoantigens:

1. Nucleotide-conjugated compounds (isopentenyl-ATP, TUBAg3-4). The TUBags were purified from mycobacterial lysates and are found in the cytosolic fractions (Constant et al., 1994, 1995; Lang et al., 1995; Poquet et al., 1996a, b). They are γ-derivatives of uridine triphosphate (X-γUTP) and thymidine triphosphate (X-γTTP). The identity of the X moiety was recently reported by Belmant et al. as being 3-formyl-1-butyl-pyrophosphate, which resembles and likely is related to isopentenyl pyrophosphate (IPP) (Belmant et al., 1999b). These TUBag compounds have been shown to be active in the nanomolar range (1000-fold more reactive than IPP), and the authors suggest that they could account for most of the γδ T-cell-stimulating activity recovered from mycobacteria.
2. Pyrophospho- and phosphomonoesters (IPP, monoethylpyrophosphate or MEPP, 2,3 diphosphoglycerate, glycerol 3-phosphate, TUBag1-2, and MALAg 1-2). IPP was isolated from mycobacteria (*Mycobacterium fortuitum*, *Mycobacterium smegmatis*) extracts and culture filtrates and described as the first structurally identified natural ligand for human γδ T cells (Morita et al., 1995; Tanaka et al., 1994, 1995, 1996). Observations by Morita et al. (Morita et al., 1999) also indicate IPP synthesis by *E. coli*, and *Y. enterocolitica*. A related compound was also identified in *P. falciparum* and is called MALAg. Vγ9Vδ2 T cells are also reactive to surface antigens on human B-cell tumors (Daudi cells). However, the relationship between phosphoantigens and "tumor antigens" for γδ T cells has not yet been clarified. The explanation for the broad reactivity of γδ T cells to a variety of microorganisms and tumor cells could be the recognition of the ubiquitous metabolites such as the prenyl pyrophosphates.

> Isopenthenyl pyrophosphate is the precursor in the synthesis of a number of important molecules including cholesterol and its derivatives (steroid hormones, vitamin D, bile salts, lipoproteins), and in the synthesis of terpenoids that modify signaling molecules in growing mammalian cells. These metabolic intermediates are conserved between prokaryotic and eukaryotic cells. Furthermore, the presence of IPP in mammalian cells suggests that γδ could potentially recognize autoantigens. It has been proposed that either differential concentration (higher in infected than in noninfected cells) or differential subcellular localization (cytoplasm vs. phagosome) of IPP could explain γδ T-cell discrimination between infected and healthy cells. More recently, it has been hypothesized that the basis of this discrimination between infected and noninfected cells is the recognition of metabolic intermediates (IPP precursors) that are produced through a biochemical pathway exclusively present in bacteria (Rohmer pathway) (Sicard and Fournie, 2000). The recently identified 3-formyl-1-butyl pyrophosphate is the favorite candidate as IPP precursor to elicit specific γδ T-cell activation and to target the response to infected cells (Belmant et al., 1999b).

How phosphoantigens are recognized by γδ TCR is not fully understood (Fig. 8.1). Although intracellular processing may not be required, cell-surface presentation and possibly extracellular processing may play a role in optimal recognition of ligands by γδ TCR (Belmant et al., 2000). Phosphoantigen recognition is TCRdependent and not restricted by MHC. Experiments with TCR-blocking antibodies and TCR transfection of the Vγ9Vδ2 TCR cDNA into TCR negative Jurkat cells have demonstrated involvement of the Vγ9Vδ2 TCR in phosphoantigen recognition (Lang et al., 1995; Morita et al., 1995). Furthermore, specificity of prenylphosphate recognition seems to be conferred by the combination of a particular CDR3 in the Vγ9 chain and the Vδ2 chain. Thus, γδ TCR junctional diversity may be responsible for differentiating among different phosphoantigens, and could be the basis for the discrimination between foreign and self-prenyl phosphates.

The existence of presenting molecules and cell-surface processing for phosphoantigens are issues that are still poorly understood (Fig. 8.1). Lack of restriction to known antigen-presenting elements (classical MHC class I or II and nonclassical CD1a, 1b, or 1c) was demonstrated by use of deficient mutant cells, transfection experiments, and use of blocking antibodies (Tanaka et al., 1994). Experiments with fixed cells, TAP- or DM-deficient cell lines, and TAP2-deficient patients demonstrated that antigen uptake and intracellular processing by antigen-presenting cells (APC) were not required for γδ T-cell activation by phosphoantigens (Fournie and Bonneville, 1996; Tanaka et al., 1994). Cell-cell contact does appear to be required and the lack of evidence for direct binding of the TCR to phosphoantigens (direct recognition) points toward the existence of an as yet unknown presenting molecule. Moreover, reactivity to phosphoantigens was lost by chemical substitution with nonhydrolyzable groups in

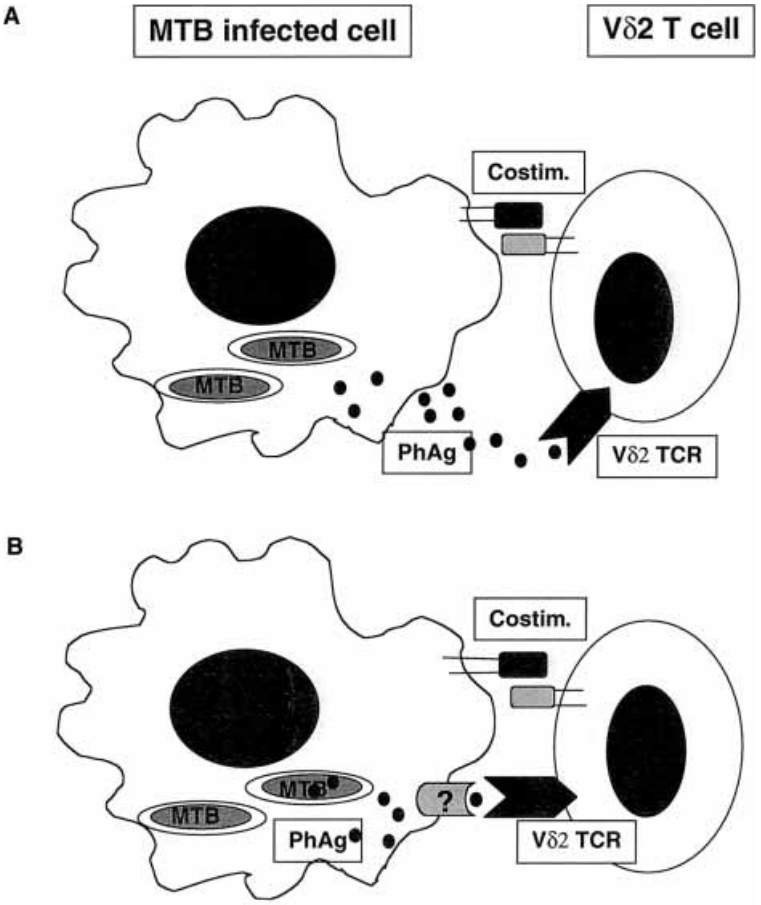

Figure 8.1. Recognition of phosphoantigens by Vγ9Vδ2 T cells. Different models of phosphoantigen recognition have been proposed based on studies with mycobacteria such as *M. tuberculosis* (*MTB*). (**A**) Phosphoantigens (*PhAg*) are secreted by intracellular MTB or originate as a product of MTB breakdown. Once phosphoantigens escape the phagocyte, they interact directly with the TCR (*Vδ2 TCR*). In this case, recognition takes place without involvement of intracellular processing or presentation by antigen-presenting cells (*APC*). Upon interaction with TCR, hydrolysis of phosphate takes place and the γδ T cell is activated (not shown). In this model, the role of phagocytes is limited to providing costimulation (*Costim.*). (**B**) MTB is taken up by phagocytes and after degradation inside phagosomes, phosphoantigens traffic inside the cytoplasm. They become stably associated with the surface of the APC, mediated possibly by an antigen-presenting molecule (yet unidentified = *?*) and interact with the γδ TCR (*Vδ2 TCR*). Hydrolysis of phosphate (P) from the phosphoantigen takes place (surface processing) allowing optimal T-cell activation. Phagocytes also provide costimulation (*Costim.*) in addition to a presenting molecule.

synthetic analogs, indicating that the phosphate moiety is a potential substrate for enzymatic processing (Belmant et al., 1999a, 2000). It is tempting to hypothesize that this enzymatic processing could happen on the surface of APCs. Therefore, surface processing and presentation may constitute the paradigm of γδ TCR of phosphoantigens.

Recognition of MHC Class I-Related Molecules, MICA, and MICB by Intraepithelial Human Vδ1$^+$ T Cells

The nature of the antigens that stimulate Vδ1 IELs differs entirely from the phosphoantigens that stimulate peripheral blood Vγ9Vδ2 T cells. Likewise, the biological implications of these responses are different. Intraepithelial intestinal Vδ1 T cells recognize self-antigens that are distant relatives of MHC class I molecules (MHC class Ib molecules), called MICA and MICB, and function as stress-induced antigens (Bahram et al., 1994, 1996). The genes for these proteins are located close to the HLA-B locus and their expression is under control of promoters that respond to heat shock. MHC class Ib are not antigen-presenting molecules but instead function as markers of cell stress or transformation (Groh et al., 1998). MICA/B were detected in cultured and freshly isolated carcinomas and increased frequencies of Vδ1 T cells have been reported in epithelial tumors, especially in those positive for MIC antigens (Groh et al., 1999). In addition, Groh and colleagues (1998) showed that γδ T cells expanded from a human MICA$^+$ colon carcinoma were able to recognize human B lymphoma cells transfected with MICA in a TCR-dependent manner without antigen processing or presentation of peptide ligands. It has been reported recently that recognition of MICA may involve not only γδ TCR but also the activating NKG2D receptor, resembling recognition of MHC class I by αβ TCR and CD8. Recognition of stress-inducible MICA antigens by γδ T cells may constitute an important surveillance mechanism in defense against tumors and other epithelial injuries.

ROLE OF γδ T CELLS IN THE IMMUNE RESPONSE TO INFECTIOUS PATHOGENS

Large numbers of microorganisms have been implicated in the activation of γδ T cells. In humans, a role for γδ T cells has been proposed in infections with the following pathogens: *S. aureus* and *Streptococci* infections (Abo et al., 1990; Mochizuki et al., 1994; Munk et al., 1990; Rust et al., 1990); *Listeria monocytogenes* (Guo et al., 1995; Munk et al., 1990); *Brucella* (Bertotto et al., 1993); *E. coli* (Morita et al., 1995); *Salmonella* (Abo et al., 1990; Hara et al., 1992); *Yersinia* (Young et al., 1997); *M. tuberculosis* (Barnes et al., 1992; Havlir et al., 1991; Inoue et al., 1991; Ito et al., 1992; Janis et al., 1989; Kabelitz et al., 1991); *Mycobacterium leprae* (Uyemura et al., 1991); *Plasmodia* (Behr and Dubois,

1992; Behr et al., 1996; Goerlich et al., 1991; Goodier et al., 1992; Ho et al., 1994; Roussilhon et al., 1990); *Leishmania* (Modlin et al., 1989); HSV-1 (Bukowski et al., 1994; Maccario et al., 1995), EBV (De Paoli et al., 1990; Hacker et al., 1992); CMV in kidney transplant recipients (Dechanet et al., 1999a, b); and HIV-1 (Autran et al., 1989; Boullier et al., 1995; De Maria et al., 1992; De Paoli et al., 1992; Hinz et al., 1994; Margolick et al., 1991).

M. tuberculosis infection is one of the diseases in which the role of γδ T cells has been studied extensively. *M. tuberculosis* induces γδ T-cell expansion dominated by the Vγ9Vδ2 subset (Kabelitz et al., 1991). Although initially HSP 65 was thought to be the antigen responsible for this reactivity, it became clear that small phosphate-containing molecules are the main antigens recognized by γδ T-cell responses in tuberculosis (see "Antigen Repertoire and Recognition").

M. tuberculosis is an intracellular pathogen that infects and resides within mononuclear phagocytes. Thus, *M. tuberculosis* is an excellent model to study the interaction between γδ T cells and accessory cells. As pointed out before, phosphoantigens can be recognized in an MHC-independent manner, not requiring uptake or presentation. However, γδ T-cell responses to intact *M. tuberculosis* bacilli depend on accessory cells. Both monocytes and alveolar macrophages were found to be efficient accessory cells (Balaji and Boom, 1998; Boom et al., 1992). It has been difficult to determine whether accessory cells function as mere sources of costimulatory signals or if they also process and present antigens to γδ T cells. In contrast to the proposed "surface processing model" (discussed in "Recognition of Phosphoantigens by Human Vγ9Vδ2$^+$ T Cells"), studies with *M. tuberculosis* bacilli indicated that mycobacterial antigens require uptake, are processed intracellularly, and are stably presented on surface of monocytes (Balaji and Boom, 1998).

As potent sources of IFN-γ and competent cytotoxic effector cells, γδ T cells may play an important role in tuberculosis infection, complementing the protective function of CD4$^+$ T cells. Some studies have tried to address the in vivo role of γδ T cells in the human immune response to *M. tuberculosis*. Evidence for a role in protection was supported by demonstration of lower expansion of γδ T cells in response to heat-killed mycobacteria in patients with pulmonary or miliary tuberculosis (Barnes et al., 1992). Although γδ T cells were found in the lung of tuberculosis patients, their proportion was not increased in comparison to αβ T cells (Schwander et al., 1996). Other studies demonstrated strong correlation between the absence or loss of Vγ9Vδ2 T cells and manifestations of disease (decrease in number and impaired function of blood and lung Vγ9Vδ2 T cells in patients versus controls) (Li et al., 1996).

The greater ability of PPD$^+$ persons compared to PPD$^-$ people to activate γδ T cells in response to *M. tuberculosis* brings additional support for a role of γδ T cells in protective immunity. Furthermore, vaccination with BCG increased in vitro expansion of Vγ9Vδ2 T cells after stimulation with *M. tuberculosis* antigens (Hoft et al., 1998).

More recently, evidence for an important role of γδ T cells in protection against *M. tuberculosis* came from an in vitro study. Dieli et al. demonstrated

that Vγ9Vδ2 T cells reduce the viability of intracellular *M. tuberculosis*. This inhibition of mycobacterial growth was the consequence of γδ T-cell cytotoxic activity mediated by a granule exocytosis mechanism that resulted in killing of the intracellular pathogen (Dieli et al., 2000).

γδ T CELLS IN HIV INFECTION

HIV is a retrovirus that infects primarily T cells and macrophages. As the infection progresses, $CD4^+$ T cells are eliminated resulting in an immunocompromised state. The final stage of the disease is characterized by development of opportunistic infections and malignancies, responsible for the high mortality of this infection. In healthy adults, 3–10% of peripheral blood T cells are γδ TCR^+ cells, of which 70–90% carry the Vγ9 and Vδ2 TCR and only 20–30% express the Vδ1 chain. The main alteration of the γδ T cell subset in HIV-1-infected persons is the selective expansion of the Vδ1 γδ T cells and a decrease in Vγ9Vδ2 T cells, resulting in the reversal of the normal Vδ2/Vδ1 ratio. In fact, Vδ1 γδ T cells constitute >70% of the γδ T lymphocyte repertoire in HIV-infected patients (Autran et al., 1989; Boullier et al., 1995; Chervenak et al., 1997; de Paoli et al., 1991a, b; Hinz et al., 1994). Importantly, these γδ T-cell alterations correlate with overall level of immunodeficiency and occur before the development of opportunistic infections, suggesting that it is not linked to a challenge with environmental antigens. (De Paoli et al., 1991). This increase in Vδ1 γδ T cells in peripheral blood is a selective feature of HIV-1 infection because it was not detected in other viral infections such as HSV-1, HSV-2, or hepatitis B or C. Rossol and colleagues suggested that this selective Vδ1 expansion may support a role of γδ T cells in immunosuppression and progression of HIV infection (Rossol et al., 1998). In addition to circulating γδ T-cell changes during HIV-1 infection, alterations in IELs γδ T cells have been demonstrated. Nielssen et al. showed high numbers of γδ T cells in duodenal mucosa in HIV^+ patients; however, progressive loss of γδ IELs was associated with a short survival expectancy in late-stage AIDS (Nilssen et al., 1996).

γδ TCR Repertoire and γδ T-Cell Surface Markers in HIV-Infected Persons

Flow cytometric analysis of peripheral blood lymphocytes from HIV-infected people have demonstrated that, although the total number of γδ T cells is preserved or even increased, the proportion of $V\delta1^+$ cells is augmented (Autran et al., 1989; Boullier et al., 1995; Chervenak et al., 1997; de Paoli et al., 1991a, b; Hinz et al., 1994). De Maria et al. demonstrated that the alteration in the ratio Vδ2/Vδ1 in peripheral blood preceded the inversion of the CD4 to CD8 ratio in HIV-infected individuals. The same relative increase in Vδ1 expression was detected in bone marrow mononuclear cells in HIV^+ patients (Rossol et al., 1998) In addition to the characteristic Vδ1 usage, changes in Vγ chain usage

were also reported in HIV^+ persons. Hinz et al. reported that whereas 80% of all γδ T cell from HIV^- persons express Vγ9 chain, only 10% of the γδ T cells in HIV^+ persons reacted with the anti-Vγ9 monoclonal antibody (mAb). Most γδ T cells in HIV-1 infected individuals were Vγ9/Vγ2/Vγ3 or Vγ4 negative and thus they expressed either Vγ5 or Vγ8, which are rarely found among peripheral blood lymphocytes in healthy adults (Hinz et al., 1994). More recently, analysis of Vγ gene usage by both flow cytometry and polymerase chain reaction (PCR) revealed that all VγI genes (i.e., Vγ2, 3, 4, 5, and 8) were expressed in higher percentages in HIV^+ when compared with HIV^- persons. In contrast, VγII gene (Vγ9) expression was markedly reduced. In addition, no preferential association between Vδ1 with a particular Vγ gene was observed (Wesch et al., 1998).

Characterization of Vδ1-J1 junctional diversity in peripheral lymphocytes of HIV-infected persons demonstrated the stability of the Vδ1 repertoire over time and suggested the absence of a CDR3-dependent antigenic selection of this subset (Boullier et al., 1995).

The analysis of Vδ1 CDR3 size distribution and comparison of Vδ1-Jδ junctional sequences showed both restricted and unrestricted patterns in HIV^+ and HIV^- persons. Thus, the Vδ1 predominance in HIV^+ persons does not seem to be a consequence of an HIV-1 specific clonal expansion (Boullier et al., 1997; Hinz et al., 1994).

Phenotypic analysis of γδ T cells from peripheral blood and bone marrow at different stages of the HIV infection showed that a large fraction of Vδ1 T cells from HIV^+ individuals display the phenotype of activated cells. Vδ1 T cells from HIV^+ persons frequently coexpress CD8 and CD38 and CD45RO and HLA-DR, although expression of the IL-2 receptor (CD25) was not higher than that found on Vδ1 T cells from HIV^- controls (Boullier et al., 1995; Rossol et al., 1998). HLA-DR, CD8, and CD45RO expression did not vary with the stages of HIV spectrum. In addition to activation markers, Vδ1 T cells from HIV-infected individuals showed an increased capacity to expand in response to IL-2 (Boullier et al., 1995).

Origin of the Expanded Circulating Vδ1 T Cells in HIV Infection

The origin of the expanded blood Vδ1 T-cell pool in HIV infection is unknown. Comparative analysis of phenotype and TCR repertoire in lymph node (LN) and blood showed that these Vδ1 populations differ in activation marker expression and in Vδ1 repertoire (Boullier et al., 1997). Both populations expressed CD38, however LN γδ T cells did not express CD45RO (memory marker). Furthermore, there were differences in the Vδ1-Jδ1 CDR3 size distribution between the two γδ T-cell populations, with the LN Vδ1 T cells showing a Gaussian CDR3 size distribution. This suggests that the lymph node is not a site of Vδ1 T-cell activation in HIV-infected persons.

One interesting hypothesis postulates that blood Vδ1 T cells originate in the

gut from Vδ1 IELs (Boullier et al., 1997). Vδ1 T cells from these two sites shared features such as restricted Vδ1-Jδ1 rearrangements, decreased expression of CD62-L, and absence of CD28 expression. In addition in vitro studies have shown that HIV infection of monocytes can disrupt endothelial monolayers, thereby increasing endothelial permeability (Dhawan et al., 1995). Furthermore, intestinal epithelia has been shown to support HIV and simian immunodeficiency virus (SIV) replication and could constitute a repository of virus (Veazey et al., 1998). Interestingly, Vδ1-Jδ1 T cells increase in intestinal epithelia and there may be an association with survival in advanced HIV patients (Nilssen et al., 1996). Taken together, these reports indicate that the activated Vδ1 T cells detected in peripheral blood of HIV-infected persons may represent a subset derived from γδ IELs. The recently described reactivity of γδ IELs to nonclassical MHC class Ib molecules, and their capacity to lyse cells that express stress-induced antigens may have important implications in HIV pathogenesis (Groh et al., 1998, 1999).

Other authors have demonstrated an increase in absolute number and percentage of γδ T cells in bronchoalveolar lavage recovered from HIV patients with $CD8^+$ alveolitis (Agostini et al., 1994, 1995). In this study, the pulmonary T-cell subset expressed Vδ2 and CD45RO and 30% coexpressed CD8. Interestingly, the increase in the Vδ2/Vδ1 ratio in the lung correlated with decreased Vδ2/Vδ1 cell ratio in peripheral blood and may reflect redistribution of Vδ2 cells to specific organs during HIV infection. The sequestration of Vδ2 T cells in lung could lead to an increase in the proportion of Vδ1 T cells in the peripheral blood (Agostini et al., 1994).

A different explanation for the origin and activation of Vδ1 T cells in peripheral blood of HIV^+ individuals was proposed by Hyjek and colleagues (Hyjek et al., 1997). These authors found that Vδ1 clones isolated from HIV^+ individuals produced IFN-γ and showed cytotoxic activity in response to lymphoblastoid cell lines (LCLs) and peripheral blood B cells from HIV^+ patients but not to LCLs or B cells from HIV^- controls. Furthermore, the stimulatory ability of B cells from HIV^+ patients was associated with expression of activation markers (CD38) and Ig production, suggesting that Vδ1 expansion might be a consequence of functional and phenotypic alterations of B cells. Interestingly, Vδ1 expansion could be triggered by recognition of a specific ligand on the surface of chronically activated B cells (see below), and thus Vδ1 T cells may have a role in immune surveillance for B cell lymphomas, which develop with increasing frequency in end-stage HIV infection.

In conclusion, different explanations have been proposed for the characteristic Vδ1 expansion in HIV infection: a) Vδ1 T cells may be derived from γδ IELs; b) the increase in Vδ1/Vδ2 ratio in blood is a consequence of redistribution of the Vδ2 subset to specific organs; and c) Vδ1 expand as a consequence of B-cell activation and expression of γδ stimulatory antigens on the surface of B cells. Further studies will need to determine which of these three models is the most likely explanation for the marked expansion of Vδ1 T cells.

Antigen Repertoire and Functions of Vδ1 T-Cell Subset in HIV Infection: Implications for the Immunopathogenesis of AIDS

The antigen specificity of Vδ1 γδ T-cell responses in HIV infection is unknown. Recognition and lysis of B-cell lines obtained from HIV^+ donors by Vδ1 T cells derived from infected persons have been reported (Hyjek et al., 1997). Thus, Vδ1 T cells may react to ligands expressed by B cells as a consequence of their chronic activation during HIV-1 infection. In addition, the diversity of Vγ chain usage, the extensive junctional diversity, and lack of MHC restriction of the LCL and B-cell-specific Vδ1 clones in HIV-1-infected individuals suggest a superantigen-like ligand. Interestingly, infection of T-cell lines with HIV or SIV induces cell-surface expression of an HSP62 family member and CD50 (Blast-1 or TCT.1) (Bartz et al., 1994). Consistent with this finding, stress-inducible MICA and MICB molecules are expressed on tumor cells, suggesting that cellular ligands expressed in states of chronic cellular activation (like tumors or viral infections) could be recognized by γδ T cells. (Ananthan et al., 1986; Jindal and Malkovsky, 1994).

γδ T cells isolated from HIV^+ donors display cytotoxic activity and secrete cytokines (Fig. 8.2). Both Vδ1 and Vδ2 γδ T-cell subsets from humans or

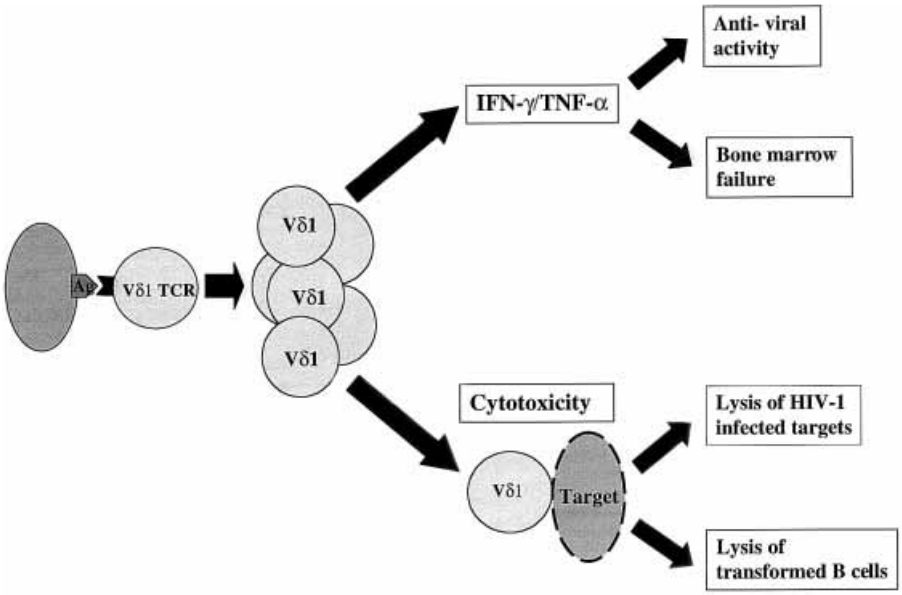

Figure 8.2. Antigen recognition and functions of Vδ1 T cells in HIV-1 infection. Vδ1 T cells recognize HIV-1-induced superantigens, B-cell-derived antigens, or MIC antigens (*Ag*) and undergo activation and expansion. Vδ1 T cells secrete cytokines or display cytotoxic activity. These functions could have not only an immunoprotective role but also implications in the immunopathogenesis of HIV-1 disease.

nonhuman primates have been shown to elicit lytic activity to a variety of targets, such as HIV- and SIV-infected cells and anti-CD3$^-$ coated HL60 cells (Boullier et al., 1997; Wallace et al., 1994, 1996). Kozbor et al. suggested that Vδ1 T cells respond to HIV and may be cytotoxic for HIV-infected cells (Kozbor et al., 1993). Recently, a substantial number of Vδ1 T cells from HIV-infected donors was found to express TiA-1 and perforin, and Vδ1 T-cell lines derived from these patients display cytotoxic activity (different from lymphoteine-activated killer [LAK] and natural killer [NK]-like activities) for anti-CD3$^-$ coated HL60 targets (Boullier et al., 1997).

Along with their potent cytotoxic activity, Vδ1 T cells also secreted IFN-γ and tumor necrosis factor (TNF)-α. The role of these cytokines in HIV immunopathogenesis is not well established, and could have regulatory, protective, or deleterious functions during the course of the HIV infection (Hacker et al., 1995; Macchia et al., 1993; Sarin et al., 1995). For example, Geissler and colleagues demonstrated that both IFN-γ and TNF-α could be implicated in the pathogenesis of bone marrow failure during HIV infection (Geissler et al., 1996). They showed that depletion of CD8$^+$ Vδ1 T cells, but not of CD4$^+$ or CD8$^+$ αβ TCR$^+$ T cells, increased the pluripotent and lineage-committed progenitor cells colony-forming units—granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM), burst-forming units—erythroid (BFU-E), and colony-forming units—granulocyte-macrophage (CFU-GM) in HIV-infected persons. Furthermore, neutralizing anti-IFN-γ and anti-TNF-α antibodies restored colony growth for CFU-GM in HIV infection, although direct cell contact between Vδ1 T cells and hematopoietic progenitor cells also contributed to hematopoietic failure (Dobmeyer et al., 1998; Geissler et al., 1991, 1992, 1996).

Antigen Repertoire and Functional Capabilities of Vδ2 T-Cell Subset in HIV Infection: Implications for the Immunopathogenesis of AIDS

As mentioned above, the two most significant alterations in γδ T cells during HIV infection are selective expansion of Vδ1 T cells associated with a decrease in Vγ9Vδ2 T cells (Boullier et al., 1995).

Vγ9Vδ2 T cells respond to mycobacterial phosphoantigens (see "Antigen Repertoire and Recognition"). Wallace and co-workers showed that γδ T-cell clones isolated from SIV$^-$ primates or HIV$^-$ donors lyse either SIV- or HIV-infected targets in an MHC-unrestricted manner (Wallace et al., 1994, 1996). This lytic activity was restricted to Vγ9Vδ2$^+$ T cells and absent in Vδ1-bearing T cells. The fact that Vγ9Vδ2 T cells from HIV$^-$ donors without previous exposure to the viral antigens showed lytic activity against virus-infected cells suggests that the antigens recognized may be of cellular origin (stress-induced) and could constitute an important anti-HIV mechanism in healthy HIV$^-$ persons. In addition, phosphoantigen-reactive Vγ9Vδ2 T cells have been shown to recognize and lyse HIV-1-infected cells inducing apoptotic death through a perforin/granzyme pathway (Poccia et al., 1997). This could be an important

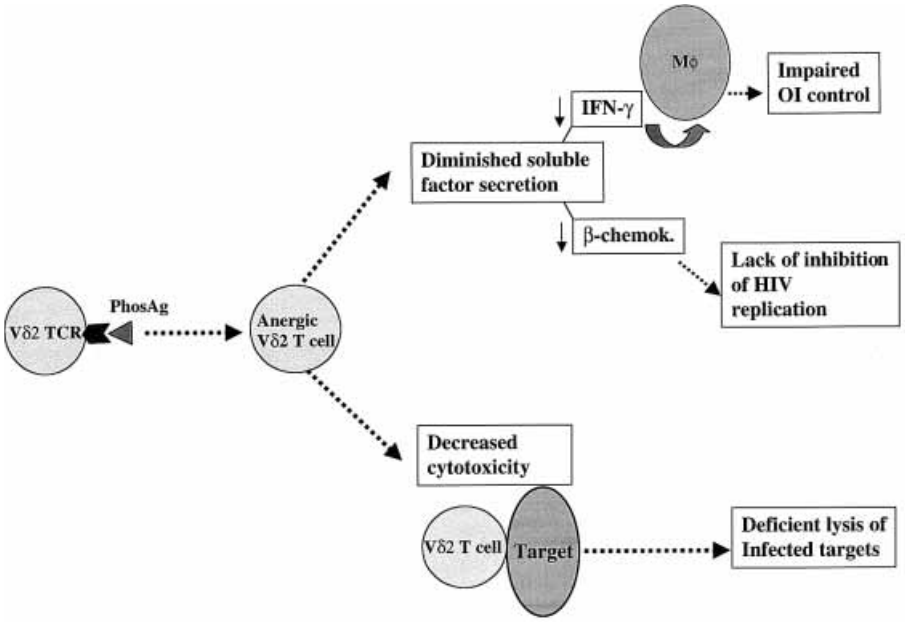

Figure 8.3. Dysregulation of Vγ9Vδ2 T-cell function in late stages of HIV-1 infection. Vγ9Vδ2 normally recognize phosphoantigens (*PhosAg*) and undergo activation and expansion. These responses are down-regulated in the late stages of HIV-1 infection (indicated by broken arrows). Vδ2 T-cell anergy results in poor IFN-γ secretion, deficient macrophage (*Mϕ*) activation, and subsequent lack of control of opportunistic infections (*OI*). β-chemokines (*β-chemok.*) display antiviral activity, and their deficient production in HIV^+ patients may contribute to lack of control of HIV replication. In addition, deficient cytotoxic activity results in impaired elimination of infected targets.

antiviral function of γδ T cells, in particular for their ability to respond quickly in an HLA-unrestricted manner. It has been demonstrated that Vγ9Vδ2 from HIV-infected patients show defective proliferative responses against phosphoantigens and low cytotoxicity against Daudi cells when compared with Vγ9Vδ2 T cells from non-HIV-infected individuals (Chia et al., 1995; Wallace et al., 1997). Defective function of γδ T cells may play a role in the increased risk for oportunistic infections during late-stage HIV infection (Fig. 8.3).

The second important function of Vγ9Vδ2 T cells is cytokine secretion. Nonpeptide-reactive Vγ9Vδ2 T cells showed a predominant Th1 phenotype, secreting IFN-γ upon stimulation with minimal IL-4 secretion (Follows et al., 1992; Morita et al., 1991). In HIV-infected individuals, especially in patients with opportunistic infections, it has been demonstrated that Vγ9Vδ2 T cells are deficient in production of IFN-γ after phosphoantigen stimulation (Fig. 8.3) (Martini et al., 2000; Boullier et al., 1997; Garcia et al., 1997). This defect in IFN-γ secretion was not reversed by IL-12 or IL-15, two potent Th1-inducing cytokines (Boullier et al., 1997; Garcia et al., 1997). This contrasts with the ability of IL-12 to restore αβ T-cell responses in HIV infection (Clerici et al.,

1993). A cause of failure in phosphoantigen-reactive γδ T-cell responses in HIV infection has been attributed to a lack of IL-2 secretion resulting from $CD4^+$ T-cell deficiency Th1 subset. Wesch et al. showed that the Vγ9Vδ2 defect was restored by low concentrations of exogenous IL-2 (Wesch et al., 1998). Thus, the authors attributed the γδ T-cell defect to absence or functional anergy of *Mycobacteria*-reactive Th1 CD4 T cells. In contrast, Poccia and colleagues showed that the Vγ9Vδ2 population from HIV-infected individuals displays selective anergy for mycobacterial antigens that cannot be reversed by IL-2. This defect correlated with a defect in the α-chain IL-2R (CD25) expression (Poccia et al., 1996). γδ T-cell functional alteration was observed in HIV^+ persons before CD4 counts dropped and was associated with an increase in Vδ1 T cells (Boullier et al., 1995; Poccia et al., 1996). Three possible explanations have been proposed to explain the Vγ9Vδ2 cytokine and other functional defects in HIV infection (Boullier et al., 1999):

1. Decreased CD25 expression by anergic Vδ2 T cells.
2. Abnormal expression of KIRs, which transmit negative signals upon TCR engagement with the specific ligand (Carena et al., 1997).
3. Chronic activation (''superantigenic-like activity'') and functional anergy by unknown ligands. This is related to the persistent activation of the immune system caused by HIV and subsequent accumulation of blast cells, which can be potent stimulators of Vδ2 T cells (Halary et al., 1997). Candidates for this chronic stimulation are cellular antigens induced or modified by viral infection. It has been suggested that this functional anergy may represent a regulatory mechanism to down-regulate autoimmune Vδ2 T-cell reactions (Boullier et al., 1999).

Another important functional capability of γδ T cells is secretion of antiviral factors. Poccia and colleagues reported that phosphoantigen-reactive Vγ9Vδ2 T cells suppress HIV replication in vitro by releasing C-C chemokines (MIP-1a, MIP-1-b, RANTES, and SDF-1) and suggest that γδ T cells may have a role in natural protection against HIV infection (Poccia et al., 1999). Similarly, a model of simian immunodeficiency showed that γδ T cells secrete β-chemokines that bind to CCR5, preventing SIV (or HIV) infection (Lehner et al., 2000).

Role and Significance of γδ T Cells in HIV Infection

The overall role of γδ T cells in HIV infection remains unclear. The presence of functional γδ TCR T cells may be important because they normally do not express CD4, an essential component for viral fusion. HIV-reactive γδ T cells could respond to infected cells without the threat of infection by the T-cell tropic virus. However, the significance of Vδ1 and Vδ2 T cells may differ giving the many biological differences between these two subsets. For example, although circulating Vδ1 T cells are normally underrepresented and nonfunc-

tional, their expansion in HIV^+ persons in addition to their activated phenotype, cytotoxic capability, and secretion of cytokines may suggest a protective role in HIV disease. The ability of Vδ1 T cells to respond to activated, EBV-infected, or transformed B cells may be relevant in controlling the development of lymphoma in AIDS patients. However, Vδ1-derived cytokines could also have a deleterious role in view of their implication in bone marrow failure in HIV^+ patients. Future studies will need to clarify whether the function of Vδ1 T cells is deleterious or protective in HIV-infected individuals.

The decreased number and function of Vδ2 T cells may contribute to the increased susceptibility to *M. tuberculosis* and *M. avium* infection in HIV-infected persons. The ability of phosphoantigen-reactive Vγ9Vδ2 T cells from healthy individuals to kill HIV-infected targets, along with secretion of cytokines and antiviral factors (chemokines), suggest that these cells may have a role in early immune defenses against HIV (Fig. 8.3).

CONCLUSION

Great advances in γδ T-cell biology have been made in the last 10 years. One of the most interesting aspects of γδ T cells is their ability to recognize non-peptidic antigens in a MHC-unrestricted manner. γδ TCR recognition of microorganism-derived phosphoantigens in a MHC-unrestricted manner may allow them to respond quickly to invading organisms, serving as a link between innate and adaptive immune responses. γδ T cells may also recognize stress-induced cell-derived antigens, playing a role in immune surveillance not only in infection but also in malignant transformation. The main alteration of γδ T cells in HIV-1 infection is inversion of the Vγ2/Vδ1 ratio, accompanied by down-regulation of Vδ2 responses to phosphoantigens and increased activation of Vδ1 T cells. The exact role of γδ T cells in HIV infection has not yet been clearly established. Vδ2 T cells may have a protective role against mycobacterial infection in HIV^+ persons. Their impaired function may contribute to the increased susceptibility to mycobacterial infections, especially in the advanced phases of AIDS. Vδ2 T cells could contribute also to control HIV-1 itself in the initial phase of infection through release of antiviral factors and cytotoxicity of infected cells.

The activated phenotype of Vδ1 T cells indicates that these cells may be responding to stress-induced stimuli and could perform immune surveillance against malignant transformation. Vδ1 T-cell failure may contribute to increased B-cell lymphoma development in advanced AIDS. There are still many aspects of γδ T-cell basic biology that require further understanding, such as the existence of presentation molecules for their ligands, the possibility of extracellular processing for γδ antigens, and the mechanisms of cross-talk with other cells of the innate and adaptive immune system. A further understanding of these and other aspects of γδ T-cell function may help to better interpret their role in HIV infection and disease.

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9

Natural Killer Cells in HIV Infection and Role in the Pathogenesis of AIDS

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INTRODUCTION

Natural killer (NK) cells were originally defined functionally as a lymphocyte subpopulation that spontaneously lysed tumor cells, virally infected cells, and, in some instances, normal cells (Herberman et al., 1975; Trinchieri, 1989, 1995). It is generally thought that NK cells represent a first line of defense against infectious agents and tumor growth. NK cells differ from T or B cells as they do not express known T-cell receptors (TCRs) or immunoglobulin (Ig) receptors for antigen. Unlike T cells, NK cells kill target cells in a non-major histocompatibility complex (MHC)-restricted fashion (see Table 9.1 for general properties of NK cells).

NK maturation can occur in the absence of a functional thymus, but NK cells share with T cells a number of properties including the expression of CD7 and CD2 antigens and effector functions such as cytolytic activity and cytokine production. These shared properties support the notion that T and NK cells may belong to the same or related lineages. Indeed, evidence exists suggesting that functional NK and/or T lymphocytes can be derived from immature cell precursors present in embryonic liver or thymus of mice and are included in a small fraction of cells expressing the CD45 antigen (Sanchez et al., 1993). Precursors that are capable of undergoing in vitro differentiation toward mature

TABLE 9.1. Properties and Functions of NK Cells

General Properties

- Lymphocytes from healthy individuals exhibit spontaneous cytotoxicity
- NK cells isolated from human peripheral blood kill certain targets (cancer, virally infected)
- NK cells mediate ADCC through the CD16 receptor
- Antigen nonspecific
- No clonal specificity
- No MHC restriction
- No immunological memory
- Dependent on intact bone marrow but not thymus
- No expression of T- or B-cell surface receptors
- Express inhibitory and activating receptors
- Light buoyant density (separate on Ficoll)
- Defined as LGL (high plasma to nucleus ratio)—Azurophilic granules
- Constitute 3–15% of peripheral blood lymphocytes
- Do not appear to recirculate
- NK activity augmented by various stimuli (microbial, IL-2, IL-12, IL-15, interferon [IFN]-α, etc.)
- Augmented activity per cell basis; proliferation of percursors; redistribution
- NK interacts with endothelial cells (NK LFA-1—ICAM-1)
- Mature NK cells are short lived, half life 1–2 days
- After bone marrow transplantation, NK are first to reconstitute

NK Cell Functions

- Natural resistance against microbial and viral infections (important player in non-adaptive immunity)
- Readily and rapidly responsive to biological response modifiers
- Mediate killing of tumor cells in tissues by several distinct mechanisms
- Poised to produce a variety of cytokines
- Endowed with receptors that facilitate entry into solid tissues
- Exercises immunoregulatory control with respect to other immune cells

NK cells are present in the immature $CD3^-$ $CD4^-CD8^-$ thymocyte subset isolated from the postnatal thymus. Further, polyclonal or clonal populations can be isolated from human thymus that express phenotypic and functional features that are intermediate between T and NK cells (Poggi and Minagari, 1995).

Several reports have investigated the differentiation of NK cells. The lack of rearrangement of genes encoding antigen-specific receptors (Ig and TCR) distinguishes NK cells from B and T lymphocytes. Hence, mice deficient in genes encoding enzymes involved in recombination of antigen-specific receptors (e.g., RAG-2) lack both B and T lymphocytes but possess normal NK cells (Shinkai et al., 1992). In contrast, mice deficient in genes necessary for the development of lymphoid cells (e.g., Ikaros) failed to develop mature NK cells and T and B lymphocytes (Georgopoulos et al., 1994). These findings suggest the presence of

a common lymphocyte of NK cell progenitors that diverge and connect toward either the T/B or NK lineages prior to recombination of genes encoding antigen-specific receptors. The common NK lymphocyte progenitors are present within a cell population of hematopoietic stem cells present in bone marrow, fetal liver, and umbilical cord (Williams et al., 1998). NK progenitors tend to differentiate in culture conditions that contain cytokines (interleukin (IL)-2, IL-7, IL-15) and other multilineage hematopoietic growth factors (stem cell factor [SCF], Flt-3) (Brasel et al., 1996; Williams et al., 1997). The cytokine IL-15 has been implicated in NK differentiation. Addition of IL-15 alone or in combination with other cytokines results in the development of functional mature NK cells from progenitors.

Phenotypically, NK cells are characterized by the expression of low-affinity receptors for IgG (FcγRIII, CD16) and the neural cell adhesion molecules (NCAM) homologous CD56 antigen. In addition, NK cells are characterized by typical cytoplasmic azurophilic granules (large granular lymphocytes [LGL]).

NK cells kill target cells by various mechanisms. Most notably, following interaction with target cells, degranulation takes place. In these granules, several factors are present such as perforin and granzymes (Shi et al., 1992). Perforin kills target cells by necrosis via induction of pores on the target cell membrane. This mechanism of killing is rapid. Through the pores, granzyme penetrates the cells and can induce apoptosis. Another mechanism of NK killing, first reported in the mouse and in humans (Mori et al., 1997), is through the Fas ligand on NK cells and the Fas receptor on target cells (Arase et al., 1994). This Fas-Fas ligand killing induces apoptosis and takes place within 2–6 h. Also, NK cells secrete tumor necrosis factor (TNF)-α on activation and can kill TNF-α-sensitive target cells, and killing is achieved by either necrosis or apoptosis. NK cells can also kill through the TRAIL/TRAIL ligand (Bonavida et al., 1999). A fifth cytotoxic mechanism implicates a specific NK cytotoxic factor (NKCF) that is distinct from TNF-α and perforin (Wright and Bonavida, 1982). However, the molecular identity of this factor has not been determined. In addition, NK cells express the CD16 receptor and can kill antibody-coated target cells by ADCC.

Recently, several studies have been reported on the characterization of surface receptors on NK cells that are implicated in the mechanism of NK killing. An inverse relationship was generally found between the expression of class I MHC on target cells and their susceptibility to killing by NK cells. This has led to the discovery of receptors on NK cells that recognize class I MHC and the existence of an NK cell repertoire (Bottino et al., 1995). Several NK receptors have been discovered and these have been shown to serve as inhibitory receptors or activating receptors. Bottino et al. (1995) have identified inhibitory receptors on NK cells that recognize human leukocyte antigen (HLA) class I molecules, and this recognition generates a negative signal that inhibits NK cytotoxicity, thus resulting in target cell protection from lysis. Recognition of HLA class I is mediated by clonally distributed receptors, several of which have been identified and cloned.

Both inhibitory and activating NK receptor (NKR) isoforms have been described (Biassoni et al., 1996; Olcese et al., 1997), and several inhibitory and activating NKRs can be expressed at the cell surface. Inhibitory NKRs harbor intracytoplasmic immunoreceptor tyrosine-based inhibiting motifs (ITIMs), which recruit and activate the protein tyrosine phosphatases SHP-1 and/or SHP-2 (Vivier and Daeron, 1997). Activating NKRs lack ITIMs and associate with polypeptides such as DAP-12/KARAP or FcR-γ containing immunoreceptor tyrosine-based activating motifs (ITAMs), which recruit and activate the protein tyrosine kinases, Syk and ZAP-70 (Lanier, 2001; Lanier et al., 1998; Tomasello et al., 1998). As in humans, NK cells from the mouse also express receptors that recognize class I MHC and this recognition prevents the lysis of the target cells (Bennett et al., 1995; Yokoyama et al., 1995).

Although NK cells have been primarily defined by their cytolytic activity, they also perform many other functions. NK cells are potent producers of cytokines such as TNF-α, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 (Trinchieri, 1995). In vivo, NK cells play an important role in fighting infections by secretion of cytokines like IFN-γ long before T-cell activation takes place and the T cells secrete their own cytokines. IFN-γ activates phagocytic cells for phagocytosis of microorganisms (Dunn and North, 1991). The induction of cytokine production by NK cells is mediated by various nonspecific stimuli (immune complexes, target cells, microorganisms). Further, other cytokines produced by T cells and macrophages, such as IL-2, IFN-γ, TNF-α, IL-1, and IL-12, can also activate NK cells and phagocytic cells and thus both cells play a central role in innate resistance to certain microorganisms (Fernandez et al., 1999). An important new cytokine, IL-12, has been identified and it is a potent stimulator of IFN-γ secretion from NK cells and B cells. Phagocytic cells, dendritic cells, and Langerhans cells are major producers of IL-12 upon infection. Thus, IL-12 activates NK cells for IFN-γ secretion, and IFN-γ then activates macrophages. IL-12 also acts on T cells for enhanced generation of cytotoxic T lymphocytes (CTL). Further, NK cells play a direct early role in IL-12-mediated induction of T-helper cells type 1 through secretion of IFN-γ in antigen-specific adaptive immunity (Trinchieri, 1995).

Although NK-cell activity can be regulated by the expression of inhibitory and activating NKRs, NK cells can also become anergic or tolerant. For instance, NK cells in class I-deficient mice fail to reject autologous cells, and such NK cells thus acquire a state of tolerance. Raulet et al. (1995) proposes that one or more mechanisms might be involved in NK tolerance, namely: 1) specific clonal elimination of potentially autoaggressive NK sets; 2) specific anergy of the potentially autoaggressive NK set; and 3) potentially autoaggressive NK cells might be altered to eliminate their autoaggression while retaining their lytic potential. Jewett and Bonavida (1996) have demonstrated that NK cells can undergo a stage of split anergy following their interaction with target cells: a subset is programmed for cell death and another subset is functionally anergic for cytotoxicity but can be activated for proliferation and cytokine production.

NK CELLS IN HIV INFECTION

Decreased Frequency of Circulating NK Cells and Phenotypic Alterations

Several reports in the literature have documented that the frequency of circulating NK cells is decreased during human immunodeficiency virus (HIV) infection (Landay et al., 1990; Vuillier et al., 1988). However, these findings are based on the phenotypic expression of surface markers on NK cells as determined in healthy individuals. The reported deficiencies in NK numbers in HIV-infected individuals are primarily based on the identification and enumeration of circulating NK cells by the NK-associated surface markers CD16 and CD56. The majority of peripheral blood lymphocytes that mediate NK cytotoxic activity in healthy adult donors express both the CD16 and CD56 cell surface molecules (Lanier et al., 1986). Routinely, the frequency of circulating levels of NK cells has been obtained with a single surface marker like CD16 or CD56; in some instances, both markers are used simultaneously. Using these two NK surface markers, investigators have reported a decrease in NK cell number in HIV-infected individuals (Landay et al., 1990; Margolick et al., 1991). Using the CD56 marker, investigators reported a decrease in both the percentage and the absolute number of NK cells in HIV-infected individuals (Vuillier et al., 1988; Mansour et al., 1990). Clearly, studies determining the frequency of NK cells on the basis of CD16 and/or CD56 expression might be misleading inasmuch as NK cells in HIV^+ individuals exhibit significant down-modulation of the CD16 and CD56 markers, and such a population will not be accounted for in the estimated overall NK cell number.

Our laboratory has examined the expression of both CD16 and CD56 surface markers in HIV-infected individuals (Hu et al., 1995). Three-color flow cytometry was used to examine the relationship between the $CD4^+$ cell numbers and the numbers of circulating NK cells as defined by the expression of the CD16 and/or CD56 molecules in control uninfected subjects and in adults infected with the HIV type 1. The HIV-infected donors had a broad range of $CD4^+$ cell levels, thereby making it possible to correlate the degree of NK cell numerical deficiency with progressive stages of HIV-mediated disease including AIDS. The number and percentage of $CD16^+CD56^+$ NK cells, the subset that comprises $> 90\%$ of the NK cells in normal adults, was profoundly decreased in HIV disease even in subjects with high $CD4^+$ cell levels. Meanwhile, the number of $CD16^{dim/+}CD56^-$ cells, an NK population that is rare in normal adults, was elevated (median of $20/mm^3$ in uninfected controls and $64/mm^3$ in early HIV disease). Furthermore, not only were many NK cells in HIV-infected subjects negative for expression of the CD56 molecule by fluorescence-activated cell sorter (FACS) analysis, the majority also expressed reduced levels of the CD16 molecule. Some $CD56^+$ cells and virtually all $CD56^-$ cells were $CD16^{dim}$. Functional studies on FACS-sorted cells revealed little NK or ADCC activity in the $CD16^{dim}CD56^-$ cell population (Hu et al., 1995). The mecha-

nisms underlying the phenotypic changes and decrease in NK cell frequency are not clear, but our studies suggest strongly that these alterations are mediated primarily by interaction of naive NK cells with target.

Reduced Ability of NK to Secrete Factors

We have previously proposed a model of NK cytotoxic function in which the NK cells bind to the target and trigger the cytotoxic mechanism through the secretion of cytotoxic factors (Wright and Bonavida, 1982). Further, it is now clear that cytotoxicity by NK cells can be mediated by degranulation of granules containing perforin and granzymes, both of which contribute to target cell lysis (Shi et al., 1992). In a previous study, we reported that, unlike NK cells from normal individuals, NK cells from HIV-infected individuals were functionally depressed and this depression was caused in part by failure of the target cells to trigger the release of cytotoxic factors from the effector NK cells (Bonavida et al, 1986). However, the NK cells were not devoid of cytotoxic factors inasmuch as activation by IL-2 stimulated the secretion of cytotoxic factors but in quantities much less than in control NK cells. These studies thus established one mechanism of functional inactivation of NK cells in which the NK cells from HIV^+ individuals may lack appropriate receptors that signal cell activation and degranulation.

Cytotoxic Functions

NK cells exert direct cytotoxicity against NK-sensitive target cells and antibody-dependent cellular cytotoxicity (ADCC) via the FcR against antibody-control target cells.

NK Cytotoxicity. Several reports in the literature have demonstrated that NK cytotoxic function is depressed in HIV-infected individuals and this function deteriorates as a function of disease progression. Depressed NK functional activity has been observed in asymptomatic and symptomatic HIV-infected individuals and profound NK cell functional deficiency has been reported in acquired immunodeficiency syndrome (AIDS) (Bonavida et al., 1986; Liu and Janeway, 1990; Plaeger-Marshall et al., 1987; Voth et al., 1988). The reported low NK cell numbers and their decreased functioning may contribute to the susceptibility of HIV-infected subjects to HIV-related opportunistic infections, Kaposi's sarcoma, and lymphomas.

Studies on the role of NK cytotoxicity in HIV-infected adult individuals have yielded contradictory results (Blumberg et al., 1987; Fontana, 1986; Katz et al., 1987, 1988; Ljunggren et al., 1989; Ojo-Amaize et al., 1989; Sirianni et al., 1988; Tyler et al., 1990). The differences may result from the use of different strains of virus, targets cell lines, test conditions, and the addition or not of exogenous antibodies for ADCC.

During a bout of moderate physical activity, HIV-seropositive individuals

apparently have an impaired ability to mobilize neutrophils, NK, and LAK cells into the circulation. In some studies, training has also attenuated psychological stress. Given the impairment of resting immune function, the potential immunosuppression from very intensive bouts of competitive exercise must be avoided. Large-scale randomized and long-term studies of HIV are needed, comparing the therapeutic value of exercise alone with that of psychotherapy or a combined program of both (Shephard, 1998).

A study examined the role of stress hormones in the progression of HIV infections. Cortisone and adrenoiorticotropic hormone (ACTH) inhibited NK cell activity in vitro by NK from AIDS but not from normal donors. The selective inhibitory effects of cortisone and ACTH in patients with HIV infections are consistent with the model that proposes that stress-related neurohormones and/or neuropeptides may be involved in the progression of HIV infection (Nair et al., 1995).

NK cell activity, either unstimulated or stimulated with INF-α, IL-2, and LAK cell activity is suppressed in patients. The activity did not differ in patients with or without AIDS. Asymptomatic patients have a higher activity than symptomatic patients. The total $CD16^+$ cells was low in patients whereas the percentage of $CD16^+$, $CD56^+$, and $CD16^+CD56^+$ were either normal or elevated (Ullum et al., 1995).

ADCC. NK cells can mediate cytotoxicity against NK-resistant target cells by ADCC through the trigger of the FcR (CD16) on NK cells by binding antibody-coated target cells. The same individual NK cell can mediate both cytotoxic functions (Bradley and Bonavida, 1982). The ADCC function of HIV-derived NK cells was examined and was found to be normal, although the same population was deficient in NK cytotoxicity. These studies demonstrated that the CD16 FcR on NK cells is functional and is independent of the NK trigger receptor for direct cytotoxicity. Further, these studies corroborated the findings above that suggested that the lytic machinery is not completely abolished in NK cells derived from HIV^+ individuals.

MECHANISMS OF NK FUNCTIONAL INACTIVATION AND DEPLETION

Anergy

NK cells undergo a state of functional anergy once they interact with the target cells. During HIV infection, NK targets increase in frequency and, thereby, through the interaction with NK cells, the NK cells become anergic in part and in part undergo apoptosis.

Split Anergy in NK Cells from Normal Individuals. We and others have reported that NK cells lose their cytotoxic function following their interaction with target cells (Abrams and Brahmi, 1988; Jewett and Bonavida, 1995a). The

mechanism by which NK cells become inactivated has been examined. Previous reports on the inactivation of NK cells by target cells used unfractionated or purified NK cells and did not attribute whether the inactive phenotype represented all of the NK cells or it was restricted to a particular subset of the NK cells. We have established the presence of three functionally distinct subpopulations of NK cells based on their ability to recognize, bind, and kill the target cells. These subsets consist of nonconjugate-forming free cells, conjugate-forming binding cells, and killer cells (Lebow and Bonavida, 1990). Using these subsets, we demonstrated that the inactivation of NK by target cells was restricted to the NK conjugate subset, whereas the nontarget binding free NK subset was not inactivated and responded to IL-2 (Jewett and Bonavida, 1995b). Further, purified cell-sorted NK-target conjugates were divided into two fractions: in one fraction, the NK cells were dissociated from the target cells (NK_{DC}), and in the other fraction, the conjugates were not disturbed (NK_C). After incubation overnight with IL-2, the cytotoxic function of NK_C was not augmented, although a subpopulation proliferated and secreted TNF-α and IFN-γ into the supernatant. In contrast, NK_{DC} cytotoxic activity was enhanced by IL-2, but proliferated poorly and did not secrete TNF-α and IFN-γ following IL-2 activation. The phenotype of the inactive NK was $CD16^{dim/-}CD65^{2+}CD16^{2+}$. Further, a significant fraction of NK cells in the NK_C was programmed for cell death by apoptosis. These findings reveal that sensitive target cells mediated inactivation of NK cells for cytotoxicity resulting in loss of NK cells (Jewett and Bonavida, 1996). Analysis of the levels of DNA fragmentation in the free and conjugate subsets of NK cells established that the conjugate subset had the highest hypodiploid DNA population (i.e., undergoes apoptosis).

In our studies, as a consequence of their interaction with either NK-sensitive K562 target cells or ADCC target cells, the FCRγIII CD16 receptor is down-modulated. Although a direct causal relationship between CD16 down-modulation and induction of inactivation and anergy has yet to be established, several lines of evidence point to the importance of this receptor in the induction of NK cell inactivation and its role in the programmed death of NK cells. In fact, our studies with normal NK cells and NK from HIV-infected individuals showed that sorted $CD16^{-}CD56^{\pm}$ cells were functionally anergic and further that the $CD16^{-}CD56^{-}$ population underwent programmed cell death (Jewett et al., 1997).

Anergy in NK Cells from HIV-Infected Individuals. Depressed NK cytotoxicity and depletion of circulating NK effector cells have been documented in asymptomatic and symptomatic HIV-infected individuals (Bonavida et al., 1986; Katz et al., 1987; Mansour et al., 1990; Plaeger-Marshall et al., 1987; Vuillier et al., 1988). The NK cell decline might be explained by the decrease in the number of NK cells and/or by down-regulation of the expression of the NK phenotypic markers CD16 and CD56 surface antigens. In fact, we have reported that as disease progresses, the frequency of peripheral blood NK cells

with the $CD16^{+}CD56^{+}$ phenotype decreases concomitantly with an increase in the frequency of NK cells with the $CD16^{dim/-}CD56^{dim/-}$ phenotypes (Hu et al., 1995). These findings established that the reported decrease in the frequency of NK cells in the HIV infection may be partially accounted for by the down-regulation of these NK phenotypic markers.

We hypothesized, based on the presence of an inverse relationship between the frequency of circulating $CD16^{dim/-}CD56^{dim/-}$ NK cells and the cytotoxic function of NK cells, that the loss of NK functional activity was concomitant with the loss of the CD16 and CD56 markers. Indeed, we demonstrated that the cell-sorted $CD16^{+}CD56^{+}$ NK subpopulation from the peripheral blood of HIV^{+} persons retained its cytotoxic activity, whereas the cell-sorted $CD16^{dim/-}CD56^{dim/-}$ subset was poorly cytotoxic, as assessed by both the direct and ADCC systems (Hu et al., 1995).

The number and percentage of $CD16^{+}CD56^{+}$ NK cells that comprise 90% of the NK cells in normal adults is profoundly decreased in HIV-seropositive individuals, whereas the number of $CD16^{dim}CD56^{-}$ and $CD16^{dim}CD56^{+}$ NK cells is increased. Some $CD56^{+}$ cells and virtually all $CD56^{-}$ cells were $CD16^{dim}$. By analogy to our in vitro results, these in vivo studies suggest that NK cells in HIV-infected subjects interact with target cells, possibly HIV-infected cells. Although functional and phenotypic analyses of the $CD16^{dim}CD56^{-}$ NK subset in HIV individuals show depressed NK and ADCC (Jewett et al., 1990), the surface expression of CD69 is elevated in the $CD16^{dim}CD56^{-}$ cells relative to the $CD16^{+}CD56^{+}$ cells. Secretion of IFN-γ and TNF-α was significantly depressed in the presence of phorbol myristate acetate (PMA) and calcium ionophore in the $CD16^{dim}CD56^{-}$ NK subset when compared with the $CD16^{+}CD56^{+}$ NK subset. Furthermore, unlike the $CD16^{+}CD56^{+}$ NK subset, the $CD16^{dim}CD56^{-}$ NK subset did not proliferate in the presence of IL-2. The $CD16^{dim}CD56^{-}$ subset was found to have a higher percentage of fragmented DNA in the presence and absence of dexamethasone when compared with the $CD16^{+}CD56^{+}$ NK cells (Jewett et al., 1997). Furthermore, the $CD16^{dim}CD56^{-}$ subpopulation had higher levels of Fas mRNA as determined by reverse-transcriptase polymerase chain reaction (RT-PCR). Altogether, these studies indicate that the $CD16^{+}CD56^{+}$ NK subset undergoes a series of functional and phenotypic differentiation in the presence of K562 or HIV target cells that results in loss of cytotoxic function, proliferation, cytokine secretion, and death of the NK cells. Table 9.2 summarizes the properties of NK subsets from HIV-infected individuals.

Expression of NKRS

The discovery of novel inhibitory receptors (also called killer inhibitory receptors, KIRs) specific for MHC class I molecules (NKRs) has stimulated considerable interest in their possible involvement in the impairment of cytotoxic function of T cells and NK cells during HIV infection. Inhibitory NKRs do not display variability and deliver inhibitory, rather than triggering, signals.

TABLE 9.2. Properties of NK Subsets Sorted from HIV-Seropositive Donors

	$CD16^+CD56^+$	$CD16^{dim/+}CD56^-$
K562 cytotoxicity	+++	+
ADCC	+++	+
TNF-α secretion	+++	−
IFN-γ secretion	+++	−
Proliferation by IL-2	+++	−
CD69	+	++
Fas mRNA	−	++
Hypodiploid DNA	−	++

They were originally identified in NK cells and have been shown to be responsible for the ability of NK cells to discriminate between normal and abnormal cells. Interaction between NK receptors and MHC molecules expressed in normal cells leads to inhibition of NK cell cytotoxicity. In humans, NK receptors belong to three distinct molecular families namely, the Ig superfamily, and include several receptor molecules with two or three extracellular Ig-like domains specific for HLA-C (p58), HLA-B (p70), and HLA-A alleles (p140). A second group is formed by type II membrane molecules that are part of the C-type lectin superfamily. They are formed by heterodimers formed by the covalent association of CD94 and NKG2A. IL-15 and TGF-β have been shown to regulate the induction of CD94/NKG2A (Andre et al., 1999). These receptors are specific for the nonclassical HLA molecules such as HLA-E. A third class with Ig superfamily structure is represented by the LIR-1/ILT-2 molecules. They do not bind to HLA-E but bind to various HLA-1 and act as promiscuous receptors for different HLA including HLA-G (De Maria and Moretta, 2000). Despite the structural diversity of the three receptors, a common feature is a characteristic sequence in the cytoplasmic domain of most of these receptors. These receptors express the ITIM. Upon phosphorylation of the tyrosine residue in these receptors, they bind the tyrosine phosphatases, SHP1 and/or SHP2, that in turn terminate positive signals transmitted via other receptors and thus inhibiting NK cell-mediated cytotoxicity or T-cell activation (Lanier, 1998).

In humans, certain inhibitory NKR molecules with short cytoplasmic domains and the NKG2C glycoprotein do not contain ITIM but have a lysine residue in the transmembrane sequence. Several of these associate with a 16-kD phosphoprotein that is expressed as a disulfide-bonded homodimer. A protein of 12 kD was identified and called DAP12 and is expressed as a disulfide homodimer on dendritic cells, monocytes, granulocytes, NK cells, and the subset of T cells. Upon ligation, it becomes tyrosine phosphorylated and is responsible for cell activation (Lanier et al., 1998).

A report by Galiani et al. (1999) reported the up-regulation of the expression of KIRs in both T and NK cells in HIV-infected individuals. The KIRs in-

cluded p58, p70, and ILT2 as well as CD94 and NKG2A. Also, IL-10 upregulates the expression of CD94 in normal $CD56^+$ cells, suggesting that the high level of circulating IL-10 in HIV-infected individuals may be in part responsible for the overexpression of CD94 on NK cells and HIV infections. Although the expression of KIRs has been examined on NK cells from HIV-infected individuals, it is important to also investigate the expression of activating receptors.

Natural cytotoxicity receptors that trigger human NK cell-mediated cytotoxicity have been identified and have been called by Moretta et al. (2000) natural cytotoxicity receptors (NCRs). They consist of three groups:

1. NKp46 is expressed on all NK cells (both resting and activated) but absent from other cells tested. Upon crosslinking, it leads to calcium mobilization, cytotoxicity, and cytokine release. NKp46 masking by specific monoclonal antibodies blocks lysis of most target cells.
2. NKp44 is absent in fresh NK but is expressed on IL-2-activated NK cells. It is absent on T cells and other linkages. Masking of NKp44 by monoclonal antibodies partially blocks cytotoxicity.
3. NKp30 cooperates with NKp46 and NKp44 in the induction of cytotoxicity against a variety of target cells. It is responsible for killing targets not affected by NKp44 or NKp46. It is expressed on both fresh and activated NK cells.

Although the expression of NCRs has not been reported in NK from HIV-infected individuals, the balance between the expression of KIRs and NCRs and their regulation may be critical for their function and activity against HIV infection.

Apoptosis of NK Cells in HIV Infection

NK cells undergo both anergy and apoptosis following interaction with normal or infected target cells. Peripheral blood lymphocytes (PBL) were tested for functional inactivation of NK cells following ADCC. PBL samples were incubated with gp120 in the absence and presence of anti-HIV antiserum. After overnight incubation, the cytotoxic functions of the ADCC samples, as well as control samples, were determined in a 4-h assay using ^{51}Cr-labeled K562 target cells. PBL samples that mediated ADCC had considerably less cytotoxic function when compared with control samples. Likewise, the percentage of fragmented DNA was significantly greater in the ADCC samples than in control samples. The same results were obtained when gp120-coated $CD4^+$ CEM and Jurkat T cells were used as ADCC targets. The addition of IL-2 to the ADCC samples in the 4-h ^{51}Cr release assay increased the cytotoxic activity of NK cells relative to baseline cytotoxicity. The percentage of dead cells in the ADCC samples was further analyzed with two different methods, namely, trypan dye

exclusion and protease inhibitor (PI) uptake. With both methods, a significant increase in the percentage of dead cells was observed in the ADCC samples when compared with control samples. It is of interest to note that addition of anti-HIV antiserum to NK from one HIV-infected patient mediated a significant increase in the number of dead cells as determined by PI uptake. This increase might reflect the increase in HIV-infected T cells in this patient (Jewett et al., 1997).

The high percentage of dead cells and cells undergoing DNA fragmentation in ADCC may be a reflection of depletion of both NK effector cells and T cells serving as target cells. To obtain the percentage of dead cells for each subpopulation, we labeled NK cells with fluoroscein isothiocyanate (FITC) after purification and reconstituted with gp120-coated target cells in the absence and presence of anti-HIV antiserum. After overnight incubation, the level of DNA fragmentation and the percentage of cells that had lost forward-angle light scatter were calculated with two parameters, log green fluorescence and forward-angle scatter. It was found that a significant number of both NK cells and T cells in the mixed lymphocyte population had decreased in size and showed high levels of DNA fragmentation in the ADCC samples. NK cells incubated with gp120-coated CEM target cells in the presence of anti-HIV antiserum showed increased levels of DNA fragmentation in both the NK cells and the CEM targets. These experiments indicated a total loss of both NK and T lymphocytes in the ADCC samples (see Table 9.3).

Highly purified NK cells obtained from HIV-seronegative donors were cultured with gp120-coated autologous $CD4^+$ T lymphocytes in the presence and absence of anti-HIV antiserum and incubated overnight to induce antibody-dependent NK cell cytotoxicity (ADCC). The NK samples were then subjected to the 4-h ^{51}Cr release assay to assess their cytotoxic function against K562 target cells. Relative to control samples, a significant inhibition of NK function was observed in the samples where ADCC had taken place. Likewise, a significant increase in the percentage of fragmented DNA was observed in the ADCC samples when compared to control samples.

TABLE 9.3. Properties of Normal NK Cells after Interaction with the ADCC Target Cells

Properties	Control	Anti-HIV serum	gp120	gp120 and anti-HIV
CD16	+++	+++	+++	−
CD56	+++	+++	+++	+
CD69	−	−	−	+++
Cytotoxicity	+++	++	+++	+
TNF-α secretion	−	−	−	+++
IFN-γ secretion	−	−	−	+++
Fas	−	−	−	+++
DNA fragmentation	−	−	−	+++
Recovery	+++	+++	+++	++

Other Mechanisms

Recombinant and synthetic peptides derived from the HIV-1 genome corresponding to the protein envelope (Env) and internal core protein (Gag) were examined for their effect on NK from normal and AIDS. Normal lymphocytes pretreated for 24–72 h with Env-Gag, Env 487–511, or Env 647–659 significantly suppressed NK cytotoxicity but significantly stimulated proliferation. In target binding assays, lymphocytes precultured with Env-Gag specifically suppressed the target binding capacity of effector cells and decreased levels of secreted NK cytotoxic factor (NKCF). The inhibition was found in both normal and AIDS-derived cells (Nair and Schwartz, 1997).

Healthy controls show statistically significantly higher NK cytotoxic activity than either HIV-1-infected asymptomatic or AIDS groups of patients. Whereas challenge of samples from normal with IL-2, INF-α, or mixture of TPA^+ ionophore resulted in significant enhancement in NK CMC. Challenge of HIV^+, asymptomatic, or AIDS resulted in an increase that was not statistically significant. This finding suggests alterations in the mechanisms responsible for NK cells activity in HIV^+ individuals (Sepulveda et al., 1997).

NK cells mediate ADCC via activation of the CD16 FcR. The receptor is linked to the zeta chain, which regulates the cells for activation. In one study, the zeta chain level was examined and revealed significantly lower levels in NK from AIDS. The CD16 decreased ADCC cytolysis by NK cells correlated with the levels of zeta chain expressed but did not with NK cell-mediated cytotoxicity (Geertsma et al., 1999).

ROLE OF NK CELLS IN THE PATHOGENESIS OF HIV INFECTION

Depletion of $CD4^+$ T Lymphocytes

It is not clear how NK cells become inactivated and depleted following HIV infection. Further, it is not clear how NK cells may regulate the frequency of circulating $CD4^+$ cells. NK cells lose their cytotoxic function and become inactivated when cocultured overnight with K562 target cells (Jewett and Bonavida, 1995a). Inactivated NK cells release significant levels of cytokines such as TNF-α and IFN-γ, which could up-regulate the replication and expression of HIV and contribute to subsequent lysis of $CD4^+$ T cells. Significant levels of TNF-α and IFN-γ are detected in AIDS patients (Jassoy et al., 1993).

Because the properties of the NK cells observed in vitro following interaction with NK-sensitive or ADCC targets are similar to those observed in NK cells from HIV-infected persons (Hu et al., 1995), we reasoned that an in vivo ADCC reaction might be responsible in part for the functional inactivation and depletion of NK cells and destruction of $CD4^+$ T lymphocytes serving as targets for ADCC. In vivo, a significant population of circulating T cells from HIV-seropositive individuals express gp120 (Daniel et al., 1993) and antibodies to gp120 have been detected in the serum of HIV-infected persons (Amadori et

al., 1992). Thus, it was possible that in vivo ADCC takes place with NK effector cells and gp120-bearing $CD4^+$ T cells and anti-gp120 antibodies leading to apoptosis of NK cells and the destruction of $CD4^+$ T cells. This hypothesis was tested in vitro. The findings revealed that gp120-coated peripheral T lymphocytes serve as targets and are killed in ADCC. Further, the NK cells that recover from the ADCC reaction show loss of cytotoxic function, acquire the $CD16^{dim/-}CD56^{dim/-}$ phenotype (as seen in vivo), and a significant fraction of NK cells are killed by apoptosis (Jewett et al., 1997). Further studies revealed that endogenous TNF-α synthesis and secretion by NK cells play a pivotal role in the induction of functional anergy and apoptosis in NK cells (Jewett et al., 1997).

The mechanism for early immune dysfunction in HIV infection with normal CD4 counts is not clear. It may be the result of inappropriate triggering of activation-induced cell death in T cells from such individuals once stimulated with specific antigens or certain mitogens. The depletion of CD4 T cells tends to result in part by infection and by apoptosis. Several mechanisms have been postulated, which include HIV Tat shown to enhance Fas sensitivity and enhance Fas ligand production; HIV Nef shown to result in activation and up-regulation of Fas ligand; HIV Vpr shown to arrest cell cycle and also have a direct effect on the permeability of mitochondria; activation-induced cell death; gp-120/160 shown to activate T cells and also up-regulate Fas sensitivity and Fas ligand (Badley et al., 2000); and apoptosis regulatory proteins, which include cellular receptors, induce apoptosis after ligation of Fas, p55 TNF receptor I, and TRAIL/Apo-2L receptor 1 and 2. The magnitude of apoptosis observed in HIV-infected patients correlates well with stage of HIV disease in longitudinal and cross-sectional analyses (Prati et al., 1997; Samuelsson et al., 1997).

Investigations have examined the effect of binding of CD4 molecules by HIV envelope protein gp-120 and cross-linking by anti gp-120 antibody. Although this cross-linking is insufficient to induce apoptosis in normal T lymphocytes, apoptosis is observed in $CD4^+$ T cells from HIV-infected individuals. There was up-regulation of Fas and this correlated with apoptosis. Also, there was induction of the cytokines IFN-γ and TNF-α in the absence of IL-2 and IL-4. Antibodies to IFN-γ and TNF-α inhibited the up-regulation of Fas apoptosis in T cells. It is possible that IFN-γ and TNF-α influence NK cells similar to the T cells and lead to their destruction (Oyaizu et al., 1995).

Peripheral blood mononuclear cells (PBMC) of HIV-infected adults are able to lyse CD4 lymphocytes expressing gp120, the major envelope glycoprotein of HIV-1, and the effector cells responsible for the lysis were shown to be $CD16^+$ and NK cells armed with cytophilic HIV specific antibody (Tanneau et al., 1990; Tyler et al., 1989; Weinhold et al., 1998). ADCC against HIV-1-expressing CD4 lymphocytes in children at various stages of HIV infection were examined. PBMC of vertically HIV-infected children were impaired in their ability to lyse HIV-expressing CD4 lymphocytes. In contrast, PBMC of

HIV-infected adults lysed such targets. The ADCC toxicity was shown to be mediated by NK cells armed in vivo with cytophilic HIV antibodies, and the number of $CD16^+$ NK cells in HIV-infected children was normal. The inability of PBMC from children to mediate ADCC against $CD4^+$ lymphocytes expressing HIV antigen was specific as they lysed HSV-infected fibroblasts in ADCC and killed HIV-infected HUT78 cells and K562 in NK CMC. The addition of HIV-seropositive serum to PBMC of children yielded mixed results with HIV-infected $CD4^+$ T cells. Sera from children interfered with ADCC from adults, suggesting blocking factors. We suggest that the defective ADCC may contribute to the rapid disease progression often observed in this patient population.

There have been many postulated mechanisms of CD4 decline in HIV infection as the percentage of infected cells is small compared with the percentage of cells lysed. A study examined the relationship of the rate of CD4 percentage decline and circulating HIV viral loads and gp120-directed ADCC. Several analyses were made in 20 patients. The rate of CD4 percentage decline was associated with either NK or ADCC activity or circulating HIV-1 RNA. Thus, the ability to mediate gp120 ADCC, together with a significant viral load, define conditions under which CD4 destruction of cells may occur (Skowron et al., 1997).

We found that NK effector cells can mediate ADCC against HIV-coated $CD4^+$ tumor target cells (Jewett and Bonavida, 1990; Katz et al., 1987), autologous $CD4^+$ T-cell blasts (Hober et al., 1995; Katz et al., 1987), and gp120-coated circulating $CD4^+$ T lymphocytes (Jewett and Bonavida, 1996). These findings demonstrate that AIDS effector cells can mediate lysis of CEM ($CD4^+$ T-cell line) coated with HIV protein in the presence of HIV-specific antibody. Lysis was specific, as non-HIV-coated CEM or the addition of HIV-negative serum resulted in no lysis. We then examined HIV-coated peripheral blood-derived $CD4^+$ T lymphocytes as targets in ADCC. We demonstrated that, in the presence of HIV-specific antibody, HIV-coated $CD4^+$ T lymphocytes serve as targets for ADCC by NK effector cells from HIV^+ patients. The lytic activity obtained with AIDS effector cells was comparable to that obtained with normal effector cells. These results demonstrate that AIDS effector cells can mediate ADCC against HIV-coated $CD4^+$ T lymphocytes and suggest that ADCC may play a role in vivo in elimination of $CD4^+$ T cells and the pathogenesis of AIDS.

NK cells after interaction with gp120-coated cells in the presence of anti-HIV antiserum lose their CD16 surface receptor significantly. This loss of CD16 is accompanied by a decrease in CD56 surface expression and an increase in CD69, CD25, and CD96 surface expression. On the other hand, the level of $CD3^+CD4^+$ T cells is significantly decreased in the ADCC samples relative to control samples. In contrast to the $CD3^+CD4^+$ T cells, the frequency of $CD3^+CD8^+$ cells is significantly increased in ADCC samples, indicating a specific loss or depletion of $CD4^+$ cells in the original $CD3^+$ T-cell population.

Along with $CD3^+CD4^+$, a subpopulation of $CD3^+CD4^-$ cells has been noted to occur as a consequence of ADCC indicating that either masking or down-modulation of $CD4^+$ cell surface receptor in ADCC had taken place.

The ability to induce ADCC is not restricted to NK cells inasmuch as monocytes/macrophages alone were capable of lysing gpl20-coated target cells in the presence of anti-HIV antibody (Jewett and Bonavida, 1990; Jewett et al., 1990; Wright et al., 1988). Hober et al. (1995) have shown that monocytes from HIV-seropositive individuals have increased spontaneous cytotoxicity and ADCC relative to HIV-seronegative donors. Furthermore, we have also shown that monocytes obtained from normal donors are capable of lysing gpl20-coated autologous $CD3^+$ T cells, indicating that this subpopulation, similar to the NK cells, can contribute to the destruction of $CD4^+$ T lymphocytes in HIV-seropositive individuals.

Other Roles

A study examined the relationship between survival and two cytotoxic immune functions and ADCC and NK against CMV targets. Cytotoxicity mediated by PBMC from 39 severely immno-compromised patients was determined. The findings demonstrate that high base-line ADCC was associated with improved survival and a similar trend was associated with NK activity. High ADCC but not NK remained significantly associated with a lower risk of death. This finding suggests that ADCC may be an important determinant of disease progression independent of antiretroviral therapy, CD4 cell count, and HIV RNA (Forthal et al., 1999).

The critical role of viral determinants in the establishment of high viral loads is best illustrated by the requirement of an intact *nef* gene for the development of AIDS in humans. Nef is a 27–34 kD myristylated protein and is shown to increase viral infectivity, T-cell activation, CD4 down-modulation, and MHC class 1 down-regulation (Saksela, 1997). Expression of Nef in cells protects them from lysis by CTL resulting from down-modulation of HLA. The HLA A and B, but not C or E, antigens are down-modulated. Interestingly, HLA A and B are the major MHC class I encoded proteins to present antigen to CTL, whereas HLA C and E can interact with various inhibitory receptors on NK cells and can protect these cells against NK (Collins and Baltimore, 1999).

A study investigated the clinical implications of impairment levels of NK and LAK during HIV-1 infection. The study reveals low NK activity was significantly associated with higher risk of progression to CD4 T-lymphocyte depression and to death. Likewise, patients with low NK responsiveness to IFN-α tended to be at higher risk of death. This finding suggests that low LAK-cell activity and low NK-cell responsiveness to IFN-α may be important in the pathogenesis of HIV infection (Ullum et al., 1999).

The pathogenesis of AIDS is a complex and prolonged process that is affected by a variety of co-factors including the abuse of both intravenous and smoked cocaine. The exact mechanism by which cocaine facilitates this disease

is not known. There has been a suggestion that cocaine alters the function of NK cells, T cells, neutrophils, and macrophages and alters the ability of these cells to secrete cytokines. In addition, cocaine enhances the infectivity and/or replication of HIV when tested using human cells in vitro (Baldwin et al., 1998).

A study examined the role of IFN-γ production and HIV progression. IFN-γ production was measured in 223 HIV^+ and 99 healthy HIV^- individuals after 4–5 h of phytohemogglotinin stimulation. IFN-γ production was followed for a median of 2.9 years. The production of IFN-γ was highly increased in the blood of HIV-infected individuals without AIDS, but decreased in the blood of AIDS patients compared with controls. In the HIV-infected patients, the production of IFN-γ correlated with the number of $CD8^+$ T cells and $CD16^+$, $CD56^+$ NK cells. Low levels of IFN-γ production were associated with an increased risk of experiencing a CD4 count below 100 cells/mm^3 and death. This finding suggests that changes in IFN-γ seem to be related to the risk of disease progression and HIV infection (Ullum et al., 1997).

A study examined how non-MHC-restricted cytotoxicity changed with respect to progression and duration of HIV infection. The major finding was that a low percentage of number of NK cells was found in the group that has a rapid progression to AIDS compared with those progressing more slowly to AIDS. Furthermore, a significant correlation was found between the number of months from zero conversion to diagnosis of AIDS and percentages of $CD16^+CD56^+$, and $CD16^+CD56^+$ cells. This finding reveals that a low concentration of NK cells in the blood was associated with a more rapid disease progression, indicating that the defective non-MHC-restricted cytotoxicity may be associated with HIV disease progression (Bruunsgaard et al., 1997).

To perform NK functions, the NK cells must adhere, migrate, and perform their function. Normally, NK cells localize in lungs, liver, and spleen after systemic administration in rats. The NK cells are not found in peripheral lymph nodes or splenic white pulp. However, NK cells can be modulated in both lymph node and non-lymph node organs after administration of biologic response modifiers. Thus, chemoattractants are implicated. Chemotactic cytokines (chemokines) are proinflammatory mediators necessary for the recruitment of various cell types to the inflammatory sites (Rollins, 1997; Murphy, 1994). Chemokines are divided into four subfamilies: CXC (alpha), CC (beta), C (gamma), and CX3C, depending on the presence and arrangement of the first cysteine residues in the amino terminal region. Chemotaxis of NK cells has been shown to be mediated by many members such as MIP-α, RANTES, MCP-1, MCP-3, IP-10, SDF-1α, etc. (Allavena et al., 1994; Godiska et al., 1997; Hedrick et al., 1997). There is evidence that chemokine receptors are coupled to heterotrimeric G proteins in NK cell membranes. In addition, a cross-talk among the various subunits and subtypes of these G proteins is obligatory for initiating the mobility of NK cells. In addition to G protein-coupled receptors, receptor tyrosine kinases transmit intracellular signals resulting in the activation of common secondary messengers. Chemokines not

only induce the chemotaxis of NK cells but also induce the mobilization of intracellular calcium in these cells (Loetscher et al., 1996). In addition, CC chemokines induce granule exocytosis in NK cells (Taub et al., 1995). The role of chemokines in NK in HIV infection is worth studying.

NK cells produce cytokines (e.g., IFN-γ and TNF-α) and the chemokine macrophage inflammatory protein (MIP-1α) following stimulation with the combination of two cytokines, IL-15 and IL-12. Monokine and activated NK cells from normal or HIV^+ donors produce similar amounts of MIP-1α, MIP-β, and RANTES protein in vitro. Because these chemokines are HIV-1-suppressive factors, supernatants from monokine-activated NK cells inhibit HIV replication in vitro. The chemokines are in part responsible for suppression, and the other factors in the supernatants are implicated. These findings suggest the role for NK in the regulation of HIV-1 infection (Fehniger et al., 1998).

CONCLUSION

The role of NK cells in the development and pathogenesis of HIV infection is supported by several lines of evidence. It is also now recognized that NK cells play a pivotal role in both innate immunity and the regulation of adaptive immunity. Thus, means to control the frequency, activity, and functions of NK cells in HIV infection are of paramount importance in the prevention as well as in the control of the progression of the disease. At present, the biology of NK cell functions is rapidly being explored and many new developments have been witnessed during the last years, such as the characterization and expression and function of NK receptors and their role in signaling the cells for functional activity. Further, new studies in the development of NK cells from precursor cells and their differentiation by various cytokines permits a better understanding of the dynamics of NK cell-mediated differentiation in infection. Therefore, continuous investigations at both the fundamental and clinical levels of the role of NK cells in HIV infection are needed for translation of the in vitro findings into the clinic to control HIV infection.

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10

Alveolar Macrophages

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INTRODUCTION

The lungs remain an important target for human immunodeficiency virus (HIV) infection. In the absence of chemoprophylaxis or specific antiretroviral therapy, the majority of HIV-infected persons experience pulmonary complications. At this anatomical locus, alveolar macrophages (AM) represent the most abundant and principal resident immune cells. AM are critical for the initiation and maintenance of normal immune and inflammatory responses in the lower respiratory tract and are poised to serve as a first-line host defense against invading pathogens. However, whether AM function is altered in the lungs of HIV-infected persons remains somewhat controversial.

Most published studies examining the effects of HIV-1 on macrophage function have been performed on monocytes or mono-derived macrophages, with few studies specifically examining AM. For comprehensive overviews of macrophage function in HIV infection, the reader is referred to a number of excellent reviews (Agostini et al., 1993a; Twigg, 1997, 1998). As the alveolar compartment represents a unique environment in which macrophages function, extrapolation from studies performed on other cells of monocyte-macrophage lineage must be made with caution. In this regard, this chapter will focus on AM (especially human AM), highlighting data that illustrate important fundamental observations in the study of AM in the context of HIV infection, and feature data derived by flow cytometry.

CELLULAR AND MOLECULAR FEATURES OF ALVEOLAR MACROPHAGES

Several excellent comprehensive reviews of AM are available to the reader (Bezdicek and Crystal, 1997; Brain, 1990; Fels and Cohn, 1986; Lohmann-Matthes et al., 1994; Sibille and Reynolds, 1990). This section will provide a brief overview of several important characteristics and features of AM.

AM are the most abundant nonparenchymal cells in the lungs, with an estimated 50–100 AM per alveolus (Crapo et al., 1982) and account for >85% of nonadherent immune cells in the alveolar airspace (Fels and Cohn, 1986). AM are predominantly derived from circulating peripheral blood monocytes and differentiation as the monocytes traverse the alveolar interstitium under the influence of cytokines and growth factors (Thomas et al., 1976). Recruitment of monocytes into alveoli is regulated by monocyte-specific chemoattractants such as macrophage inflammatory protein-1 (MIP-1) and MIP-2 (Brieland et al., 1993; Iyonaga et al., 1994), and integrin-mediated interactions. Often referred to as a uniformly functioning population of cells, AM obtained by bronchoalveolar lavage likely represent a heterogeneous group of cells (Brain, 1988). Local proliferation accounts for only $\leq$2% of the expansion of alveolar macrophages (Bitterman et al., 1984), although AM are generally long-lived with estimated life spans of months to years (Marques et al., 1997).

AM perform a number of antimicrobial and anti-inflammatory functions. AM are an important component of the innate immune response, and may influence the nature of the acquired immune response to antigen (Fearon and Locksley, 1996). AM eliminate inhaled microorganisms, clear environmental toxins, and remove cellular debris. AM also express a broad range of secretory products and mediate a variety of essential biologic activities in the context of antimicrobial activity, acute inflammatory response and tissue repair.

Antimicrobial Function

AM are "professional" tissue phagocytes and thus require the essential components of chemotaxis, binding, ingestion, and digestion. AM are capable of chemotaxis, the directed movement along a concentration gradient (Sibille and Reynolds, 1990). Chemoattractants such as N-formylated peptides, leucotriene B4, and C5a induce macrophage migration upon binding to specific receptors expressed along the leading edge of the cell surface. AM binding of particles can be nonspecific or enhanced through interaction of specific AM receptors with native molecules or opsonins decorating the particles.

Macrophages ingest or internalize molecules and small particles (<1 μm, endocytosis), and fluid (pinocytosis) via a clathrin-associated actin-independent process, and larger particles (>1 μm) via actin-dependent processes (Anderson et al., 1990; Greenberg and Silverstein, 1993; Orosi and Nugent, 1993; Wright and Detmers, 1991). Ingestion requires the formation of lamellipodia and pseudopodia, generated by assembly and cross-linking of actin filaments. Human AM

are unique phagocytes in their dependence on an oxygen tension > 25 mm Hg for optimal phagocytosis (Hunninghake et al., 1980).

Ingestion is greatly facilitated by a variety of receptors expressed on the surface of AM. Opsonin-*independent* internalization is mediated through pattern recognition receptors such as mannose receptor (Ezekowitz et al., 1990) and scavenger receptor (Palecanda et al., 1999). These receptors recognize certain patterns of molecules expressed on the surfaces of particles or pathogens, and thus represent an important component of innate immunity. The family of complement receptors (CR1, CR3, and CR4) represent another component of the innate immune response facilitating phagocytosis by an opsonin-*dependent* process (Sibille and Reynolds, 1990; Unkeless and Wright, 1988). AM expresses CR1 receptor, which preferentially binds complement component C3b but also could bind C3bi and C4b. Opsonin-dependent internalization is also mediated through the family of Fcγ receptors, including FcγIa receptor (high affinity), FcγII (low affinity), and FcγIIIa. These well-characterized phagocytic receptors recognize the Fc domain of immunoglobulin G (IgG), and mediate phagocytosis, superoxide production, and release of cytokines such as tumor necrosis factor (TNF)-α. AM do not have receptors for IgM although IgM immune complexes can bind via C3b (Gadek et al., 1980).

Finally, digestion or killing can occur by oxidative and nonoxidative mechanisms. Intracellular release of reactive oxygen intermediate species (i.e., oxidative bust response, or respiratory burst response) is a major microbicidal mechanism employed by AM. Production of reactive oxygen intermediates is myeloperoxidase (MPO) independent. By this mechanism, the principle toxic reactants formed are hydroxyl radical and singlet oxygen, which are derived from the interaction of O_2^- and hydrogen peroxide (H_2O_2). AM could also use nonoxidative microbicidal mechanisms by expressing macrophage cationic proteins, lysozyme, and other lysosomal enzymes such as elastase, plasminogen activator, and phospholipase to digest microorganisms (Orosi and Nugent, 1993). Disorders of any of these four critical components can result in serious host defense diseases such as pneumonia (Brown and Gallin, 1988).

Alveolar Macrophage Activation

AM can become activated by a variety of stimuli, both specific stimuli through specific receptors and nonspecific stimuli. Some activation signals initiate a broad spectrum of processes, whereas other signals result in limited responses. The mechanisms of AM activation are incompletely understood, although activation results in changes in morphology and general metabolic activity, changes in the density of surface molecules, and the release of a variety of molecules, including cytokines, bioactive lipids, oxidants, lysosomal enzymes, and growth factors. Activated macrophages are bigger, with more pronounced ruffling of the plasma membrane, increased numbers of pseudopodia, and increased pinocytotic vesicles. The resting metabolic activity of AM is high and increases further with activation. Activation signals include immune complexes,

interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, lipopolysaccharides (LPS), phorbol myristate acetate (PMA), leukotriene B4 (LTB4), formyl-methionyl-leucyl-phenylalanine (f-MLP), and platelet activating factor (PAF) (Reed et al., 1987; Sibille and Reynolds, 1990), although whether such activating signals are operant in vivo in the lungs remains to be established. The consequences of activation are not the same for every activation signal, and the hierarchy of activation responses of AM has not been defined.

Macrophage Secretory Products

AM release a variety of products, including oxidants, antioxidants, growth factors, enzymes, proteases, antiproteases, and bioactive lipids, that allow AM to regulate and maintain the integrity of the lungs (Kelley, 1990; Nathan, 1987; Sibille and Reynolds, 1990; Takemura and Werb, 1984). For a comprehensive review of the spectrum of macrophage secretory products, the reader is referred to several excellent reviews (Kelley, 1990; Sibille and Reynolds, 1990). Among the more important secretory products involved in antimicrobial function are the reactive oxygen species including superoxide anion, hydrogen peroxide, and hydroxyl radical, which are oxygen metabolites of the membrane-bound NADPH-oxidase system. AM also release nitric oxide, which may participate in antimicrobial function and mediate inflammation (Kuo et al., 2000).

Cytokines and Cytokine Receptors

AM can also produce a variety of cytokines including IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, macrophage colony-stimulating factor (M-CSF), GM-CSF, and transforming growth factor (TGF)-β (Kelley, 1990; Nathan, 1987). AM also express several cytokine receptors including receptors for IFN-γ, TNF-α, IL-2, IL-4, and GM-CSF. AM function can be regulated by individual cytokines or combinations of cytokines interacting with specific surface receptors. As AM secrete specific cytokines and also express the appropriate receptor, some cytokines serve an autocrine function. A broad range of biological effects can be induced by the binding of different cytokines to their specific receptors.

Accessory Cell Function

In general, AM are less potent antigen-presenting cells compared with other macrophages such as Langerhans cells and peritoneal macrophages (Roth and Golub, 1993). AM are deficient at presenting tetanus toxoid to autologous $CD4^+$ T lymphocytes, a deficiency that may be attributed to failure to express the costimulatory molecules B7-1 and B7-2 (Chelen et al., 1995). AM may suppress immune responses by producing a variety of mediators that may inhibit T-lymphocyte proliferation such as superoxide anion, nitric oxide, leuko-

trienes, prostaglandins, vitamin D metabolites, IL-1, and TGF-β (Strickland et al., 1994; Upham et al., 1995).

Other Alveolar Macrophage Surface Receptors

AM communicate with external environment via surface receptors, which are capable of binding specific ligands and activate various signal transduction pathways to produce different biological functions. Many surface receptors have been defined in AM. (Bezdicek and Crystal, 1997). AM express CD14, the receptor for bacterial LPS, although generally lower levels compared with peripheral blood monocytes. Additional examples include receptors for transferrin, histamine, β-adrenergic agonists, neutrophil elastase, cathepsin G, α1-antitrypsin, PMA, and surfactant proteins (Lohmann-Matthes et al., 1994; Sibille and Reynolds, 1990; Shepherd, 1991). This diversity of surface receptors allows AM to respond to a vast array of environmental and local influences.

Signal Transduction Pathways

As most available studies on intracellular signaling pathways utilize monocytes or monocyte-derived macrophages, the signal transduction pathways of AM are incompletely understood. AM express many of the surface receptors examined in other mononuclear phagocytes and neutrophils. The consequences of membrane receptor triggering of AM likely involves one or more second messenger pathways.

Investigtors have recently examined specific signal transduction pathways in human AM. Recognizing the importance of the nuclear factor (NF)-κB/I-κB pathway in cytokine regulation, LPS stimulation of AM results in NF-κB activation (Mathys et al., 2000). Furthermore, LPS-mediated NF-κB induction and the release of IL-6, IL-8, and TNF from human AM is dependent on tyrosine kinase and phosphatidylcholine-specific phospholipase C, and independent of protein kinase C (Carter et al., 1998). Furthermore, two members of the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (Erk) and p38 kinase, are also necessary for LPS-mediated IL-6 and TNF transcription and cytokine release (Carter et al., 1999).

MOLECULAR AND BIOLOGICAL CHARACTERISTICS OF HIV-1

A detailed discussion of HIV-1 is included in other chapters of this book, and comprehensive reviews of HIV-1 biology are available to the reader (Fauci, 1996; Greene, 1991; Rich, 1998). This section will provide an overview of important molecular and biological characteristics of HIV-1 as these relate to AM.

HIV, a lentivirus in the Retroviridae family that infects humans, is the major cause of the acquired immunodeficiency syndrome (AIDS). The related HIV-2

can also cause AIDS, and is especially endemic to Western Africa, but is less pathogenic than HIV-1. The following comments relate primarily to HIV-1.

The infectious HIV-1 particle (virion) is a spherical/icosahedral structure. The outer coat (envelope) is a lipid bilayer primarily derived from the host cell membrane and spiked with the specific HIV-1 glycoproteins, gp120 and gp41. Contained within the spherical structure is the cone-shaped nucleocapsid, composed of the major core protein HIV p24. Within the nucleocapsid structure, the retroviral capsid contains two identical copies of HIV-1 RNA, and several critical enzymes, including reverse transcriptase (RT), integrase, and protease. Each single-stranded RNA is 9.2-kb long and rich in adenosine deoxyribonucleotide residues, distinct from host cell genes (Kypr et al., 1989). The two identical viral RNA copies are composed of both structural (e.g., *gag*, *pol*, and *env*) and regulatory genes (e.g., *tat*, *rev*). The structural genes include *env*, which is the precursor for the envelope glycoprotein (gp120 and gp41); *gag*, which is the precursor for the virion core proteins (including p24); and *pol*, which is the precursor for virion enzymes including reverse transcriptase, protease and integrase. The genes can be further characterized as essential, or required for viral replication (e.g., *gag*, *pol*, *env*, *tat*, and *rev*), and nonessential accessory or auxilliary genes (e.g., *vpr*, *vif*, *vpu*, and *nef*).

HIV-1 can infect a variety of cells expressing the appropriate surface receptors. In general, HIV-1 infects susceptible cells by interaction of the gp120 glycoprotein with receptors on the surface of host cells, namely CD4 and chemokine receptors. In general, monocytotropic isolates of HIV-1 utilize the CCR5 chemokine receptor along with the CD4 receptor, whereas lymphocytotropic HIV-1 isolates utilize CXCR4 chemokine receptor in association with the CD4 receptor. The cell tropism of HIV-1 is determined primarily by the specific amino acid residues located in the 35-amino acid V3 (loop) variable domain sequence of the gp120 molecule. In general, the initial infection with HIV-1 of susceptible subjects involves a monocytotropic isolate of HIV-1 (M-tropic or C5), whereas HIV-1 disease progression is associated with evolution of the virus to acquire more lymphocytotropic characteristics (T-tropic or CX4).

Receptor-mediated entry is the major pathway for HIV-1 infection of susceptible cells. HIV-1 gp120 binds to the CD4 receptor on the host cell surface, inducing a conformational change in the gp120 molecule, which exposes the hydrophobic fusion peptide, gp41. HIV-1 gp41 then inserts its fusion domain into the host cell membrane, bringing together the host membrane and the viral envelope, which promotes membrane fusion and the release of the HIV-1 nucleocapsid into the cell cytoplasm.

As the HIV-1 nucleocapsid enters the host cell, it is uncoated, and each HIV-1 RNA is reverse transcribed to yield a double-stranded DNA replica of the original RNA genome, although now containing tandem long-terminal repeat (LTR) regions rather than the native short-terminal repeat regions of the native HIV-1 RNA. The newly synthesized HIV-1 DNA is transported into the

nucleus and, under the direction of the viral enzyme integrase, becomes randomly integrated with host cell genome. This integrated form of HIV-1 is referred to as HIV-1 proviral DNA, and remains latent until transcription is initiated by an appropriate cell stimulus. In general, HIV-1 infection of $CD4^+$ T lymphocytes is characterized by active HIV-1 replication, whereas HIV-1 infection of resting T lymphocytes or monocyte-macrophages is predominantly latent or persistent with limited viral replication.

HIV-1 transcription is initiated by the activation and binding of host cell transcription factors such as NF-κB/Rel to specific binding sites in the HIV-1 LTR region of the viral genome. Binding to the HIV-1 LTR promotor site then activates host cell RNA polymerase II. Following cell activation, the 2.0-kb HIV-1 regulatory genes (*tat*, *nef*, *rev*) are transcribed first and transported to the cytoplasm. Under the direction of the regulatory proteins, the HIV-1 structural and enzymatic genes are then transcribed as precursors and transported to the cytoplasm where these products undergo cellular protease cleavage. The HIV-1 viral nucleocapsid components, full-length HIV-1 RNA, and HIV-1 enzymes then assemble in the cytoplasm, are transported to the inner surface of the cell membrane, and then released from the infected cells by a process of budding from cell plasma membrane, acquiring the host cell lipid bilayer as the virion coat. A variety of factors can stimulate or enhance HIV replication, including cytokines, mitogens, and infectious agents. Currently, there are no vaccines that prevent HIV-1 infection and no drugs that eliminate or cure HIV-1 infection in patients. Most of the available antiretroviral drugs that are currently used to control HIV-1 infection are targeted to inhibit the function of HIV-1 RT and HIV-1 protease, and thus limit viral replication.

In the context of AM, other important characteristics of HIV-1 isolates deserve mention. In general, HIV-1 infection of $CD4^+$ T lymphocytes results in cytotoxic effects to the cells, whereas HIV-1 infection of monocyte-macrophages are resistant to the cytopathic effects of HIV-1 infection. Evidence suggests that HIV-1 evolves differently in different anatomical compartments of the same individuals, suggesting local influences may contribute significantly to HIV-1 replication and evolution and may influence drug susceptibility. Finally, most currently available drugs require active HIV-1 replication for efficacy, and whether these agents will affect long-lived and latently infected cells such as AM remains to be established.

ALVEOLAR MACROPHAGES IN HIV-1 DISEASE

Several investigators have provided important insights into the function of AM in the lungs of HIV-infected individuals and the influence of HIV-1 infection on AM function. Much of the data has been obtained by flow cytometry methodology, which has allowed the detailed investigation of various AM parameters (Table 10.1).

TABLE 10.1. Examples of Use of Flow Cytometry to Study the Influence of HIV-1 Infection of Human Alveolar Macrophages

Parameter Measured by Flow Cytometry	References
Surface expression of receptors	
CD4 receptor	Guay et al., 1997; Park et al., 1999; Wagner et al., 1992
CD 11b receptor	Wasserman et al., 1994
CD14 (LPS) receptor	Wasserman et al., 1994
CD54 (ICAM-1) receptor	Wasserman et al., 1994
CD71 (transferrin) receptor	Wasserman et al., 1994
CC, CXC chemokine receptors	Park et al., 1999; Worgall et al., 1999
TNF receptors	Skolnik et al., 2000
MHC class II receptors	Buhl et al., 1993; Wasserman et al., 1994; Koziel et al., 1998a
Mannose receptor	Koziel et al., 1998a
Phagocytosis	Ieong et al., 2000
Oxidative burst response	Koziel et al., 2000
Intracellular expression of HIV-1 p24 antigen	Ieong et al., 2000

Characteristics of Alveolar Macrophages from HIV-Infected Persons

In contrast to alveolar $CD4^+$ T lymphocytes, which decline in number with HIV-1 disease progression (Bofill et al., 1998) similar to findings in the peripheral blood, AM numbers are preserved and may be increased in >30% of individuals (Agostini et al., 1991b). AM from HIV-infected persons with active pulmonary disease demonstrate higher proportions of monocyte-like surface markers CD11b, CD14, and CD53, suggesting cells recently recruited into the alveolar airspace perhaps in response to the infectious process (Wasserman et al., 1994).

AM from HIV-infected persons contain HIV-1 proviral DNA (Koziel et al., 1999). Previous studies suggest that 0.1–10% of AM from HIV-infected persons demonstrate evidence for proviral HIV-1 DNA (Lebargy et al., 1994), indicating latent infection. More recent studies suggest that the proportion of AM in HIV-infected individuals may exceed 10% (Koziel et al., 1999). The ability to isolate HIV-1 from bronchoalveolar lavage (BAL) cell specimens in general correlates with clinical severity and HIV-1 disease progression (Jeffrey et al., 1991; Koziel et al., 1999; Sierra-Madero et al., 1994).

HIV-1 Infection of Alveolar Macrophages

AM can be infected with HIV in vitro (Ieong et al., 2000; Salahuddin et al., 1986) (Fig. 10.1). AM express CD4 receptors as well as chemokine receptors CCR3, CCR5, and CXCR4 (Park et al., 1999) (Fig. 10.2), all recognized im-

Figure 10.1. Detection of HIV-1-infected AM by flow cytometry. AM of healthy donors were infected with HIV-1 BAL isolate in vitro and cultured for 14 days, the cells detached, permeabilized and stained with phycoerythrin (PE)-conjugated KC57 antibody (which recognizes HIV-1 p24 core antigens). Compared with a PE-conjugated isotype control, live-gating on AM revealed 87% of the AM were positive for the HIV-1 p24 antigen. For details see Ieong et al., 2000. Reproduced with permission.

portant coreceptors for HIV infection of susceptible cells. However, HIV-1 preferentially utilizes the CCR5 chemokine receptor in AM (Park et al., 1999).

HIV-1 infected AM may represent previously HIV-1-infected peripheral blood monocytes that are recruited into the lungs and differentiate into HIV-1-infected AM. Alternatively, uninfected AM may become HIV infected in the alveolar airspace from the release of HIV-1 virions from actively replicating T lymphocytes. Terminally differentiated macrophages such as AM are more susceptible to HIV-1 infection compared with peripheral blood monocytes (Rich et al., 1992). Evidence suggests that HIV-1 evolves differently in blood than in lung macrophage populations (Nakata et al., 1995), suggesting that lung-specific or local influences can influence the genetic development of HIV-1. AM may be more frequently infected with HIV-1 in pediatric patients compared with adult patients (Pearce et al., 1993).

HIV-1 Replication in Alveolar Macrophages

In general, HIV-1 infection of AM is latent, with predominantly proviral DNA detectable. HIV-1 RNA, indicative of active viral replication, can be detected in the cell-free BAL fluid in up to 16% of asymptomatic HIV-infected individuals, but is detectable in the BAL fluid in 82% of HIV-infected persons with active lung disease such as *Pneumocystis carinii* pneumonia (Koziel et al., 1999). Importantly, the levels of HIV-1 RNA greatly exceed those measured in the serum of the same patients, suggesting local HIV-1 replication in the lungs of these individuals rather than exudation from the serum (Koziel et al., 1999).

Figure 10.2. Flow cytometry histograms of CD4 and chemokine receptor expression on AM from a healthy individual. For each panel, the relative log fluorescence is plotted on the ordinate (FL1 = fluorescein isothiocyanate [FITC] and FL2 = phycoerythrin [PE]), and the cell count is plotted on the abscissa. The panels demonstrate surface labeling for (**A**) FITC-conjugated isotype IgG control; (**B**) FITC-conjugated HLA-DR; (**C**) PE-conjugated isotype IgG control; (**D**) PE-conjugated CD4 receptor; (**E**) FITC-conjugated F(ab)2 secondary antibody control; (**F**) FITC-conjugated anti-CCR3 receptor; (**G**) FITC-conjugated anti-CCR5 receptor; and (**H**) FITC-conjugated anti-CXCR4 receptor. For details see Park et al., 1999. Reproduced with permission.

Factors influencing HIV-1 replication in AM in the lungs are likely complex, but may include the presence or absence of potential pulmonary pathogens such as *P. carinii* (Koziel et al., 1999) and *Mycobacterium tuberculosis* (Nakata et al., 1997), and the relative local concentrations of substances such as Regulated upon Activation, Normal T cell expressed and secreted (RANTES) (Lane et al., 1999), surfactant protein-A (SP-A) (Guay et al., 1997), TNF-α (Lane et al., 1999; Nakata et al., 1997), and IFN (Honda et al., 1998), and perhaps influences such tobacco exposure (Abbud et al., 1995). In the context of HIV-1 latency, taken together with the longevity of AM, AM likely serve as an important reservoir for HIV-1 in the lungs, which may have important clinical implications with regard to elimination of HIV-1 (Koziel et al., 1999).

Alveolar Macrophage Function in HIV-1 Disease

Increasing evidence suggests that AM from HIV-infected individuals indeed demonstrate impaired function, especially innate immune function, although results may be greatly influenced by specific pathogens studied. Table 10.2 summarizes the reported alterations in AM function. Investigations of HIV infection on AM phagocytosis have demonstrated preserved function, enhanced function (Musher et al., 1990), or impaired function (Koziel et al., 1998a). These reported differences likely reflect the complexity of host-pathogen interactions, including differences in pathogen virulence factors as well as differences in host cell recognition and phagocytosis pathways.

Antimicrobial Function

Although monocytes from HIV-infected persons may demonstrate impaired chemotaxis to a variety of stimuli (Smith et al., 1984), chemotaxis studies of AM have not been investigated. Among other important factors, phagocytosis by AM from HIV-infected individuals may be related in part to the clinical status of the HIV-infected persons as well as to the pathogen studied. Phagocytosis of the bacterium *S. aureus* is enhanced (Musher et al., 1990), whereas for the atypical mycobacterium *M. avium* phagocytosis is unchanged compared with healthy (Denis and Ghadirian, 1994) and phagocytosis of fungi such as for *P. carinii* is impaired (Koziel et al., 1998a). Reduced *P. carinii* phagocytosis may in part be attributed to reduced AM mannose receptor expression (Fig. 10.3). Ingested organisms may be killed effectively (Cameron et al., 1994), although recent studies demonstrate impaired anticryptococcal activity by AM infected in vitro with HIV-1 (Ieong et al., 2000).

Respiratory Burst Response

The generation of reactive oxygen intermediates, an important component of antimicrobial function of AM, may be preserved in AM from HIV-infected persons (Buhl et al., 1993; Murray et al., 1985), although exogenous stimula-

TABLE 10.2. Altered Human Alveolar Macrophage Function Associated with HIV-1 Infection

Observed Alteration	References
Alveolar macrophages from asymptomatic HIV-infected individuals	
Enhanced accessory cell function	Twigg et al., 1989
Increased surface expression of class II MHC molecules*	Buhl et al., 1993
Enhanced IL-1, IL-6 release	Trentin et al., 1992; Sierra-Madero et al., 1994
Dysregulated release of IL-8, IL-10, IL-12	Denis and Ghadirian, 1994a
Enhanced spontaneous release of superoxide anion†	Buhl et al., 1993
Reduced *P. carinii* binding and phagocytosis	Koziel et al., 1998a
Enhanced *C. neoformans* fungistasis	Reardon et al., 1996
Reduced mannose receptor surface expression	Koziel et al., 1998a
Reduced TNF-receptor I and TNF-receptor II surface expression	Skolnik et al., 2000
Reduced oxidative burst response to *P. carinii*	Koziel et al., 2000
Increased release of soluble mannose receptor	Fraser et al., 2000
Alveolar macrophages from HIV-infected patients with active disease	
Enhanced *S. aureus* phagocytosis	Musher et al., 1990
Reduced *P. carinii* binding and phagocytosis	Koziel et al., 1998a
Reduced mannose receptor surface expression	Koziel et al., 1998a
Reduced TNF receptor I and TNF receptor II surface expression	Skolnik et al., 2000
Reduced oxidative burst response to *P. carinii*	Koziel et al., 2000
Enhanced TNF-α release	Israel-Biet et al., 1991; Krishnan et al., 1990; Millar et al., 1991
Increased release of soluble mannose receptor	Fraser et al., 2000
Enhanced GM-CSF release	Agostini et al., 1992
In vitro HIV-1 infection of alveolar macrophages from healthy individuals	
Reduced *P. carinii* binding and phagocytosis	Koziel et al., 1998a
Reduced mannose receptor surface expression	Koziel et al., 1998a
Reduced *C. neoformans* fungicidal activity	Ieong et al., 2000
Preserved anticryptococcal activity	Cameron et al., 1990
Enhanced HIV-1 replication‡	Abbud et al., 1995

* Human leukocyte antigen (HLA)-DR, HLA-DQ, HLA-DP.

† Noncultured cells.

‡ Smokers compared with nonsmokers.

Figure 10.3. Flow cytometry analysis for surface expression of mannose receptor on human alveolar macrophages. AM were examined from healthy, asymptomatic HIV^+ individuals with high peripheral blood CD4 counts (CD4 > 200 cells/mm^3), asymptomatic HIV^+ individuals with low peripheral blood CD4 counts (CD4 < 200 cells/mm^3), and HIV^+ patients with active *P. carinii* pneumonia (HIV^+ PCP). Each panel illustrates a representative flow cytometry profile of one subject for each of the groups examined, demonstrating positive HLA-DR staining (*dashed line*) and positive mannose receptor antibody staining (*solid line*), separated from background (*shaded curve*). The ordinate represents relative cell number and the abscissa represents relative log fluorescence intensity. For details see Koziel et al., 1998a. Reproduced with permission.

tion may be required (Murray et al., 1985). However, recent investigations suggest that specific receptor-mediated impaired oxidative burst function may be apparent in persons with advance HIV infection, related in part to mannose receptor down-regulation (Koziel et al., 2000).

Alveolar Macrophage Activation

Several investigations suggest that AM from HIV-infected individuals are "activated," expressing surface markers associated with macrophages activation such as major histocompatibility complex (MHC) class II molecules (HLA-DR, HLA-DQ, HLA-DP) (Agostini et al., 1993; Buhl et al., 1993), β_2-integrins (LFA-1, CD11a/CD18), and CD54 (ICAM-1) (Agostini et al., 1993). Although some of these observations may be attributable to active pulmonary disease, studies focusing on asymptomatic HIV-infected individuals describe

enhanced spontaneous release of superoxide anion, and increased IP-10 gene expression by AM from HIV-infected persons (Buhl et al., 1993). The finding of increased IP-10 mRNA expression, generally induced by IFN-γ (Luster and Ravetch, 1987), suggests intraalveolar exposure to IFN-γ, a recognized activator of macrophages, may account for the observed activation. Other studies have not described increased HLA-DR expression (Koziel et al., 1998a). The mechanisms for the observed activated state remain to be established, but certainly in part reflect active pulmonary disease related to HIV-1 infection (as many of the subjects studied were symptomatic).

Cytokine Release

The effect of HIV-1 infection on the release of cytokines remains uncertain and controversial. AM from HIV-infected persons may spontaneously release increased amounts of cytokines, and may also exhibit an exaggerated response to in vitro stimuli (Agostini et al., 1991a, 1992; Israel-Biet et al., 1991; Millar et al., 1991; Trentin et al., 1992; Twigg et al., 1992; Sierra-Madero et al., 1994). Although these observations may in part reflect active lung disease, these findings were also observed in certain asymptomatic individuals. TNF receptors may be influenced by HIV-1 infection, as recent observations suggest that TNF-receptor I and TNF-receptor II are both down-regulated in AM from asymptomatic HIV-infected persons (Skolnik et al., 2000). The pathological consequences may influence both host defense function as well as influence HIV-1 replication of infected cells.

Accessory Cell Function

Although antigen-presenting capacity of AM may be enhanced in HIV infection, and surface expression of MHC class II molecules may be increased, the interactions of AM with mitogen-induced T lymphocytes are similar to those in healthy individuals (Twigg and Soliman, 1994). However, the specific receptor-ligand and the role of coreceptor stimulation is poorly understood. The antigen-presenting capacity of AM from HIV-infected persons may be greater compared with healthy individuals (Twigg et al., 1989). This enhanced capacity may in part reflect AM activation, although asymptomatic individuals exhibited the greatest enhanced capacity. Alternatively, the enhanced antigen-presenting capacity may reflect elevated levels of stimulatory cytokines such as IL-1 and IL-6, or enhanced AM response to these cytokines.

Surfactant Regulation

AM participate in the processing of pulmonary surfactant components such as SP-A (Hawgood and Clements, 1990). SP-A contributes to alveolar gas exchange by lowering surface tension in the alveolar airspace, and may also participate as a nonimmune opsonin in the innate immune response. Although SP-A levels are elevated in the lungs of asymptomatic HIV-infected persons

(Downing et al., 1995) and HIV-infected persons with active pulmonary disease (Phelps and Rose, 1991), the specific role of AM in this process has not been established. Furthermore, whether elevated SP-A levels facilitate or impair AM function remains to be established, and whether facilitated SP-A-mediated binding of pathogens (Downing et al., 1995; Williams et al., 1997) contributes to pathogenesis of infection remains to be determined. Some investigations suggest that pathogens may utilize host-derived SP-A to avoid recognition by host immune cells (Koziel et al., 1998b).

Surface Expression of Receptors

Increasing evidence suggests that surface receptor expression may be altered on AM from HIV-infected persons. Surface-associated macrophage mannose receptor is reduced in asymptomatic HIV-infected individuals (Koziel et al., 1998a), whereas the soluble form of the receptor is greatly increased in the BAL fluid of HIV-infected persons asymptomatic or with active *P. carinii* pneumonia, but absent in BAL fluid of healthy individuals (Fraser et al., 2000). Recent observations demonstrate that surface TNF receptors are also reduced on AM from asymptomatic HIV-infected persons, accompanied by increased soluble forms of the TNF receptors I and II in the cell-free BAL fluid of these individuals (Skolnik et al., 2000). These observations suggest that HIV infection is associated with increased release of soluble forms of macrophage receptors. The consequences of these changes are not completely understood, although soluble mannose receptors may impair AM recognition of *P. carinii* (Fraser et al., 2000).

Signal Transduction Pathways

AM signaling pathways are poorly understood and incompletely studied. Major signal transduction pathways such as NF-κB/I-κB appear intact in AM from asymptomatic HIV-infected individuals on highly active antiretroviral therapy (HAART) therapy and TNF-α release is comparable to healthy individuals (Mathys et al., 2000).

SUMMARY

The mechanism(s) accounting for altered AM function remain to be established. Investigations featuring in vitro infection of alveolar macrophages suggest that HIV-1 infection is sufficient to account in part for the alterations (Koziel et al., 1998a; Ieong et al., 2000), and may reflect the influence of specific HIV-1 gene products or alterations in vital cellular pathways mediated by HIV-1. In the lungs of HIV-1-infected individuals, altered AM function may also reflect alterations in the local alveolar microenvironment including alterations in the cellular composition, and the relative concentrations of cytokines, chemokines, and other biomodifying agents.

CONCLUSION

Most HIV-infected individuals will experience pulmonary complications during the course of HIV-1 related disease, especially in the absence of HAART and specific chemoprophylaxis. In the lungs, AM represent the most abundant pulmonary immune cell and represent an important first-line-host defense cell. Through its capacity to serve as an essential component of innate immunity, and the capacity to mediate subsequent acquired immune response, AM are important mediators in healthy individuals. Increasingly, growing evidence suggests that in addition to CD4 T-lymphocyte depletion in the lungs (which parallels the decline in the peripheral blood), AM from HIV-infected individuals demonstrate a variety of functional alterations, all of which may impact on the clinical consequences of HIV-1-related disease. Clearly, impaired innate and altered acquired immune function may impair critical functions and lead to susceptibility to opportunistic pathogens and infection and inability to adequately clear pulmonary infections.

Although much has been learned in recent years, many unanswered questions related to AM function in HIV-1 infection remain. Clearly, studying AM rather than peripheral blood–derived macrophages is important. Continued use of powerful tools, including flow cytometry, will allow a greater understanding of the function of these important cells, and hopefully lead to novel therapeutic interventions such as immunomodulation of these cells to assist in host defense function.

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11

Dendritic Cells Ferry HIV-1 From Periphery into Lymphoid Tissues

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INTRODUCTION

Dendritic cells (DC) were first described in 1868 by Langerhans in the skin, but it was not until 25 years ago that their function as potent stimulators of primary immune responses was recognized (Steinman and Cohn, 1973). Initially, progress into the characterization of DC was slow because of their low frequency, lack of markers to distinguish them from the abundant monocytes and macrophages, and the problems involved in purifying them. Research into their immune-regulatory function was greatly facilitated by the discovery that DC could be grown in culture from proliferating progenitors or nonproliferating precursors such as monocytes (Romani et al., 1994; Sallusto and Lanzavecchia, 1994).

DC are important sentinels of the immune system and are therefore essential for the onset of a strong immune response against incoming pathogens, such as human immunodeficiency virus (HIV)-1. However, besides being responsible for eliciting an HIV-1-specific response, it is now becoming evident that DC also participate in the dissemination of HIV-1. Sexual transmission via any of the oral, rectal, and vaginal mucosa is the most common route for infection with human HIV-1 worldwide. Although the nature of the immunological responses at these sites may differ, dissemination of HIV-1 could be facilitated by

a common cell-type: the DC. Various groups have shown that DC residing in blood, skin, or mucosal surfaces indeed play an important role in this process (Cameron et al., 1992b; Patterson et al., 1994; Pope et al., 1994; Spira et al., 1996). Immature DC are the first cells targeted by HIV-1 since they act as sentinels against invading pathogens. DC are directly infected by HIV-1 or capture the virus, and subsequently transport it from the periphery into the lymphoid tissues, where DC-bound HIV-1 is transmitted to $CD4^+$ T cells, the ultimate target of HIV-1. Research has mainly focused on studying HIV-1 infection of $CD4^+$ T cells, as the site for viral replication. However, a better understanding of DC as the initial target for HIV-1 in viral dissemination and chronic infection, as well as their function in eliciting immunity against HIV-1, is essential in the successful development of preventative and therapeutic strategies. Recent reviews have extensively described the immunological functions of DC (Banchereau and Steinman, 1998; Hart, 1997). We will here focus on the facets of DC biology important in HIV-1 dissemination and pathogenesis and incorporate recent findings that shed light on the mechanism by which HIV-1 subverts the migratory and immune function of DC to establish a persistent infection of $CD4^+$ T cells.

DENDRITIC CELLS AS PROFESSIONAL ANTIGEN-PRESENTING CELLS

DC are professional antigen-presenting cells (APC) that are able to efficiently stimulate both naive T and B cells. Both T and B cells need to be activated to perform their specific functions to elicit an efficient immune response against an invading pathogen, which ultimately results in clearance of the pathogen and to antimicrobial resistance. B cells, the precursors of antibody-secreting plasma cells, directly recognize native antigen presented by DC, which leads to B-cell activation. In contrast, T cells only recognize peptides bound to major histocompatibility complex (MHC) class I and II molecules. Peptides presented by MHC class I are recognized by cytotoxic $CD8^+$ T cells (CTL), whereas peptides bound by MHC class II interact with helper $CD4^+$ T cells. Once activated, the helper T cells have profound immunoregulatory effects; they interact with other cells, such as B cells, to induce antibody secretion, macrophages that subsequently release cytokines, and CTL that destroy pathogen-infected cells. Activation of naive T cells requires a second signal in addition to the recognition of peptide-MHC complexes (Mellman et al., 1998); this involves adhesion and costimulatory molecules, which are essential for prolonged and efficient interaction between T cell and DC, resulting in an efficient immune response. The professional status of DC as APC is derived from several unique features (Table 11.1), one of which is its ability to provide signal 2; nonprofessional APC, like B cells, monocytes, and macrophages, lack the ability to elicit signal 2 and are therefore only capable of stimulating previously activated T cells.

DC progenitors migrate from blood into peripheral tissues, where DC act as

TABLE 11.1. Unique Features of DC Defining Them as Professional APC

Unique DC features
In vivo localization
Antigen capture
Antigen processing
Specific migratory ability
Antigen presentation
Costimulatory molecules
Activation of naive T cells

sentinels against invading pathogens (Fig. 11.1). Upon pathogen infiltration, resident DC, as well as DC precursors from blood, migrate specifically to the site of inflammation and sample the pathogens. Captured pathogens are efficiently processed to an array of peptides for presentation at the cell surface on MHC class I and II molecules. DC migrate after pathogen capture to lymphoid tissues to present foreign peptides to T cells and activate antigen-specific T cells.

DC are such efficient APC because they exist in two functionally different forms, generally classified immature and mature. Both forms have unique abilities that are necessary for their different functions. Immature DC are highly efficient at antigen capture, internalization, and processing, but poor at

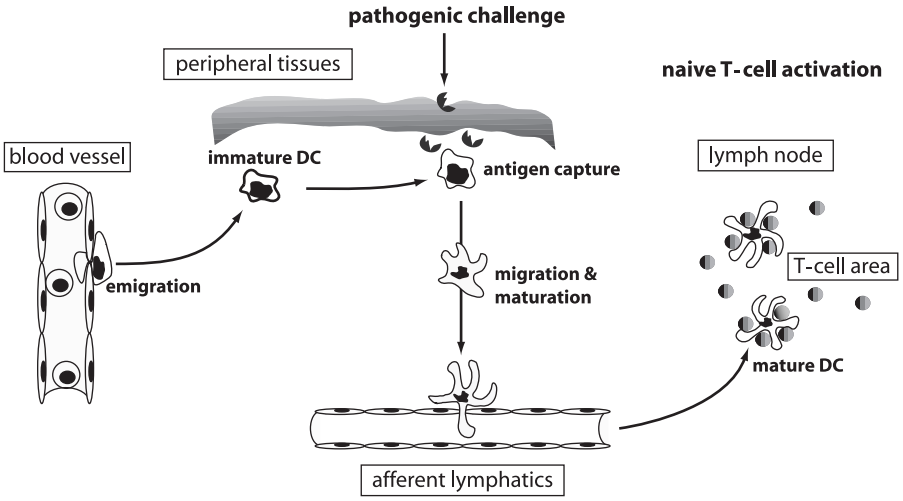

Figure 11.1. DC capture foreign pathogens, migrate, and activate T cells. DC precursors emigrate from the blood into the peripheral tissues where they differentiate into immature DC that patrol these tissues. Immature DC migrate toward sites of pathogen entry and capture antigens, after which they mature and migrate through the lymphatics into the lymphoid compartments. At these sites, the mature DC interact with resting T cells, resulting in antigen-specific T-cell activation and thus the initiation of an efficient immune response.

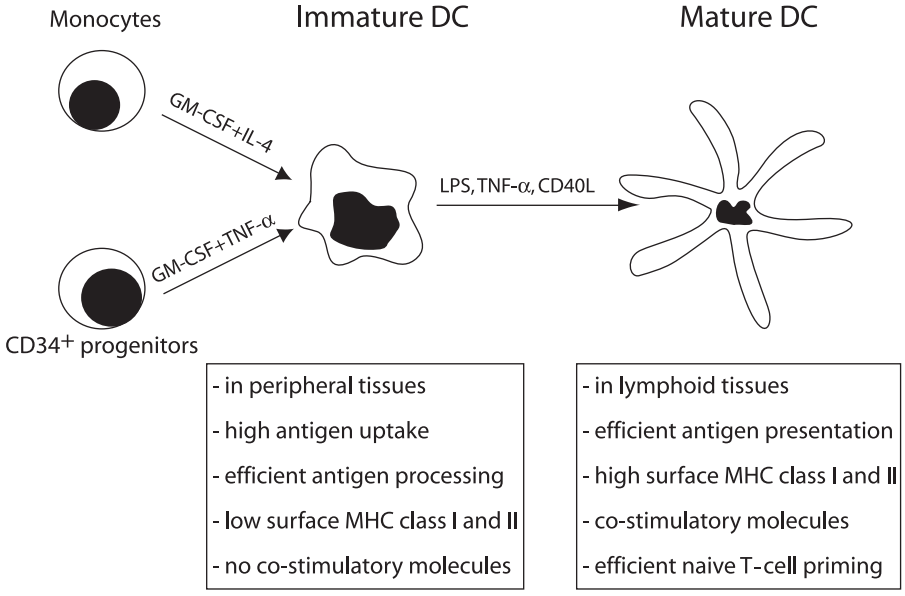

Figure 11.2. The different stages of the DC life cycle are mediated by cytokines in vitro. Immature DC are cultured in vitro from monocytes or $CD34^+$ progenitors in the presence of cytokines, maturation is induced by CD40 ligation, the bacterial cell-wall component lipopolysaccharide (*LPS*) or tumor necrosis factor-α (*TNF-α*). Immature and mature DC have different distinct characteristics, which are required for their specialized functions.

stimulating T cells. Conversely, the mature form is equipped with an extremely efficient antigen-presenting machinery, but is poor at uptake and processing (Fig. 11.2). Immature and mature DC specifically migrate to those sites where they can perform their functions optimally. Stimulatory cytokines released at sites of inflammation induce the immature DC to mature and migrate specifically to lymphoid tissues through the afferent lymph. Phagocytic and antigen-processing activities are down-regulated during maturation, and the expression of costimulatory and adhesion molecules involved in efficient antigen presentation to T cells are up-regulated. Therefore, DC transport an antigenic representation of the invading pathogen to the T-cell areas of lymphoid tissues, where they are ideally positioned to interact with many naive T cells and activate those that are antigen-specific. The peptide-MHC complexes are extremely stable, enabling a thorough screening by T cells.

MOLECULAR MAKE-UP OF IMMATURE AND MATURE DENDRITIC CELLS

The elucidation of methods of culturing DC in vitro at a defined maturation state from either monocytes or bone marrow progenitors in the presence of cyto-

TABLE 11.2. Differential Expression of Receptors During the Two DC Life Cycles

Immature DC	Mature DC
Phagocytic molecules	Antigen presentation molecules
DEC-205	MHC Class I and II
Macrophage mannose receptor	Stimulatory molecules
Fc receptor	CD80, CD86, CD83
Complement receptor	Adhesion molecules
Antigen processing/loading molecules	DC-SIGN (CD209)
HLA-DM	ICAM-1 (CD54)
Proteases	p150,95 (CD11c)
DC-LAMP	
Adhesion/migration molecules	
DC-SIGN (CD209)	

kines greatly improved our understanding of DC biology (Fig. 11.2). Monocytes treated with the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 differentiate into pathogen capturing immature DC (Romani et al., 1994). Several factors, such as whole bacteria, the microbial cell-wall component lipopolysaccharide (LPS), cytokines, such as IL-1 and tumor necrosis factor (TNF)-α, and CD40 ligation drive their differentiation into mature DC (Fig. 11.2).

Immature DC express a variety of molecules to mediate efficient intake of pathogens by endocytosis (Table 11.2), including C-type lectins, such as DEC-205 and the macrophage mannose receptor (Hart, 1997). These receptors bind heavily glycosylated antigens via their C-type lectin domains such as those commonly found on the surface of pathogens (Drickamer, 1995), and have a high turnover at the cell surface for efficient internalization of bound antigen. Immature DC contain specialized MHC class II-rich compartments, where processed antigen is efficiently loaded as peptides onto MHC class II molecules. During maturation of DC, the stable complexes of these MHC class II-rich compartments are discharged at the surface, where they remain for several days enabling screening by $CD4^+$ T cells. Recognition of these complexes by T cells and their subsequent activation requires the formation of a specialized junction between these cells, the so-called immunological synapse, that is generated by carefully orchestrated recruitment of specific adhesion receptors into the contact site to strengthen DC–T-cell contact (Geijtenbeek et al., 2000b; Grakoui et al., 1999; Monks et al., 1998). We have recently demonstrated that DC use the DC-specific ICAM-3- and HIV-1-receptor DC-SIGN (CD209) to provide the initial contact by interacting with ICAM-3 on the naive T cell. This interaction provides positional stability and facilitates engagement of the T-cell receptor (TCR) with peptide-MHC class II complexes. TCR-triggering initiates strong adhesive LFA-1 (CD11a)-mediated interactions with ICAM-1 (CD54) on DC,

and interactions of the costimulatory molecules CD80, CD83, and CD86 on DC provide the necessary signals (signal 2) for efficient activation of the T cell.

DC also present pathogen-derived peptides on MHC class I molecules to activate CTL. When DC are themselves infected with viruses, the MHC class I molecules are loaded in DC similarly as in other cells. The cytosolic viral proteins synthesized in the DC are processed into peptides and the peptides are translocated from the cytosol into the endoplasmic reticulum, where they bind to MHC class I molecules. The peptide-MHC class I complexes are translocated to the cell surface for scrutiny by CTL. DC are also able to obtain peptides for MHC class I presentation by processing apoptotic bodies from infected cells (Mellman et al., 1998).

Thus, the different forms of DC can be distinguished by differences in cell-surface expression of molecules involved in these functions (Fig. 11.2, Table 11.2). Most notably, immature DC express low levels of MHC class II on their cell surface. In contrast, mature DC express high levels of MHC class II and costimulatory molecules that are essential for efficient antigen presentation.

DENDRITIC CELL MIGRATION AND CHEMOKINE ATTRACTION

A central feature of DC is their capacity to migrate. DC progenitors migrate from blood into peripheral tissues where immature DC act as sentinels against invading pathogens. Upon pathogen infiltration, residing immature DC as well as DC precursors from blood migrate specifically to the site of inflammation, sampling foreign antigens. After antigen capture and processing, immature DC mature and migrate specifically to lymphoid tissues through the afferent lymph. In lymphoid tissues, mature DC specifically localize in the T-cell areas where they are ideally positioned to interact with naive T cells and activate antigen-specific T cells (Fig. 11.1).

The nature of the circulating blood cells that give rise to immature DC in peripheral tissues is unclear. Circulating monocytes represent nonproliferating precursors that differentiate in vitro to immature DC in the presence of cytokines (Romani et al., 1994; Sallusto et al., 1996). The factors governing the differentiation in vivo are unclear. It has recently been shown that, after trans-endothelial migration, monocytes differentiate into immature DC 2 days after phagocytizing particles, supporting a role for monocytes as blood DC precursors (Randolph et al., 1998, 1999).

Other populations of possible DC precursors have been identified in peripheral blood (Geijtenbeek et al., 2000c; O'Doherty et al., 1994; Weissman et al., 1995a), which could give rise to immature DC in peripheral tissues. These DC precursors are present in very low amounts in blood ($<0.5\%$ of total peripheral blood mononuclear cells) and extensive studies are therefore limited by their paucity. One such DC precursor expresses MHC class II, but lacks the specific lineage markers and surface activation molecules found on other leukocytes, and is probably en route to becoming tissue DC (O'Doherty et al., 1993; Thomas

et al., 1993). Another blood-borne population represents mature $CD83^+$ DC (O'Doherty et al., 1994; Thomas et al., 1993; Weissman et al., 1995) possibly nonlymphoid resident DC in transit to lymphoid tissues. Recently, we have identified two DC precursor populations in blood that express the DC-specific ICAM-3- and HIV-1-receptor DC-SIGN, but differ in CD14 expression (Geijtenbeek et al., 2000c); these populations probably represent different stages of DC precursors en route to seeding peripheral tissues as immature DC.

DC precursors are recruited from the blood to peripheral tissues constitutively to replenish the tissue or in response to inflammation. Tethering to the blood vessel endothelium is an essential prerequisite for the exit of DC precursors from the blood, and is necessary for subsequent firm adhesion and extravasation. The DC-specific adhesion receptor DC-SIGN mediates this initial tethering of DC-SIGN-positive cells along ICAM-2 (Geijtenbeek et al., 2000c), an endothelial ligand abundantly expressed on both blood and lymphatic vessels. The transendothelial migration of DC increases upon activation of endothelial cells, leading to an increase in the egress of DC precursors, and is mediated by DC-SIGN/ICAM-2 interactions. DC-SIGN is rapidly up-regulated on monocytes in the presence of GM-CSF and IL-4 (Geijtenbeek et al., 2000b); thus, DC-SIGN up-regulation by cytokine mediators may induce migration of precursor DC from blood into the periphery. The presence of DC-SIGN-positive DC precursors in blood further supports the hypothesis that, under physiological circumstances, DC-SIGN/ICAM-2 interactions mediate rolling along endothelial linings and transmigration of DC into the periphery (Geijtenbeek et al., 2000c). These DC-SIGN-positive DC precursors could be poised to exit the blood at inflammatory sites, allowing rapid recruitment of these cells to sites where their surveillance function is needed. The high expression of DC-SIGN on immature DC in the peripheral tissues, and the expression of ICAM-2 on lymphatic vessels, further supports a central role for the DC-SIGN/ICAM-2 interaction in DC-specific migration from blood into the periphery, whether inflamed or not, and subsequently of immature DC via the lymph into lymphatic tissues.

Navigation of DC from blood into tissues and to their final destination, the T-cell area of the lymphoid tissues, is governed by the action of chemokines. Chemokines are a superfamily of chemotactic proteins that can be divided in four groups on the basis of their cysteine structural motifs. Most chemokines fall in two families, the CXC group, which is mainly active on neutrophils and lymphocytes, and the CC group, which is active on multiple subsets of mononuclear cells, including DC.

It is becoming clear that distinct chemokine-chemokine receptor pairs are responsible for the different navigational steps of DC. The CC chemokines, such as IL-8, macrophage inflammatory protein (MIP)-1α, and Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES), induce chemotactic and transendothelial migration of immature DC in vitro. The two main receptors for these chemokines, CCR1 and CCR5, are expressed by immature DC; they enable the first step of DC migration from blood into inflamed tissues

where pathogens must be captured. It has recently been shown that the HIV-1 envelope glycoprotein gp120 can also induce a chemotactic response in immature DC via CCR5, resulting in specific migration toward HIV-1 in vitro (Lin et al., 2000). The CXC chemokines mediate the second stage in the DC-specific migration process, from inflamed tissues through the lymphatics and to the T-cell areas of the lymph nodes. The migrating DC are maturing and down-regulate CCR1 and CCR5, but up-regulate receptors for constitutive chemokines such as CXCR4 and CCR7 (Sallusto et al., 1998), whose chemokines, SDF-1 and SLC, are expressed in lymphoid tissues. Thus, the rapid and strict regulation of chemokine receptor expression on DC, depending on their differentiation stage, plays a vital role in the migratory capacity of DC.

EXPRESSION OF HIV-1 RECEPTORS ON DC

Three distinct HIV-1 receptors are expressed on DC: CD4, the chemokine receptors CCR5 and CXCR4, and DC-SIGN. CD4 and the chemokine receptors are also found on T cells, B cells, and monocytes, but the recently identified HIV-1 gp120-binding receptor DC-SIGN is expressed only on DC (Fig. 11.3*A*) (Geijtenbeek et al., 2000a, b). The primary HIV-1 receptor CD4 has been detected on different DC populations, albeit at lower levels than on $CD4^+$ T cells (Granelli-Piperno et al., 1996; Rubbert et al., 1998). $CD4^+$ DC populations have been identified in blood (Weissman et al., 1995a) and in vivo in mucosal tissues that are involved in sexual HIV-1 transmission, such as cervix and uterus. Langerhans cells, the DC population that resides in the skin epidermis as well as in the mucosal epithelium, are also positive for CD4 (Wood et al., 1983).

The chemokine receptors CCR5 and CXCR4 have been identified as the predominant receptors for HIV-1 entry into target $CD4^+$ T cells (Littman, 1998). The primary coreceptor for M-tropic HIV-1 is CCR5 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996), whereas CXCR4 is the coreceptor for T-tropic HIV-1 (Feng et al., 1996). The natural ligands of these HIV-1 coreceptors, the inflammatory chemokines RANTES, MIP-1α, MIP-1β (Combadiere et al., 1996; Samson et al., 1996) and SDF-1, prevent infection by obstructing HIV-1 *env*-mediated membrane fusion and thus viral entry (Alkhatib et al., 1996; Bleul et al., 1996; Deng et al., 1996). Predominantly M-tropic HIV-1 strains are responsible for the initial dissemination events after sexual transmission of the virus; the location of DC in the peripheral mucosal tissues has greatly enhanced the study of their involvement in the selection of only M-tropic HIV-1 strains, and the predominant expression of CCR5 on DC has therefore been of particular interest. Expression of both CCR5 and CXCR4 has been detected on in vitro cultured DC, but the expression of both depends on the maturation state, DC subtype, and manner of isolation; variations are observed between DC studied in vitro and in vivo. Both blood- and monocyte-derived DC express CCR5 and CXCR4 (Ayehunie et al., 1997; Delgado et al., 1998; Dittmar et al.,

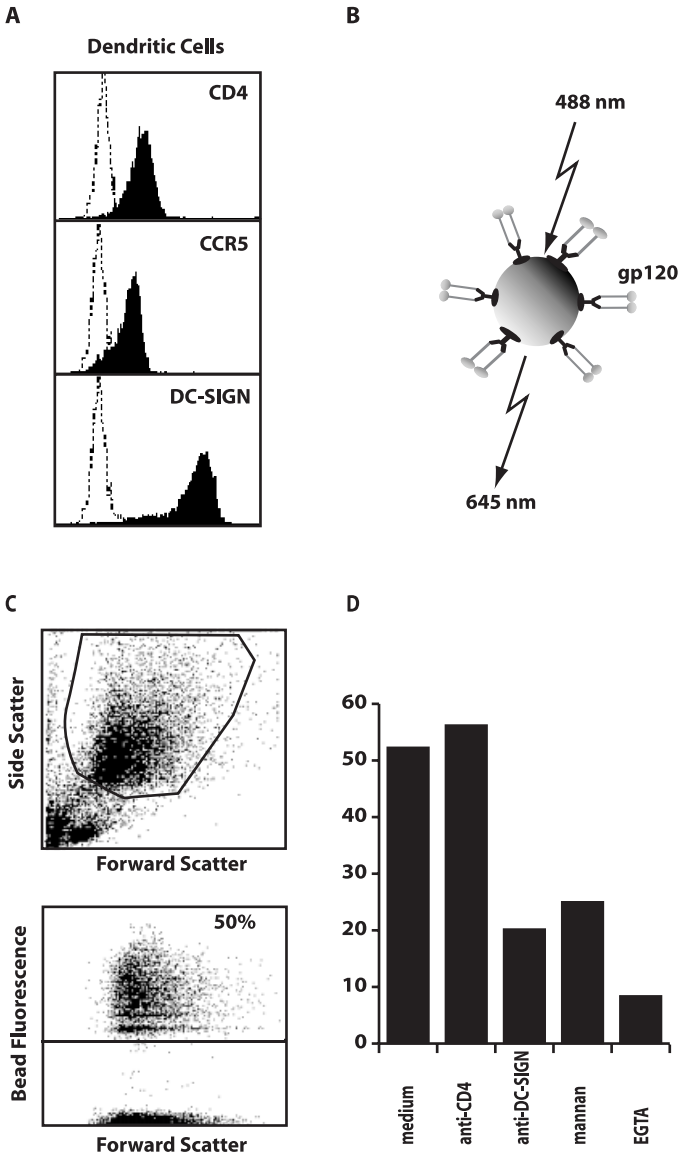

Figure 11.3. DC-SIGN on DC binds HIV-1 gp120 with high affinity, in contrast to CD4 and CCR5. DC express high levels of DC-SIGN and low levels of both CD4 and CCR5 as determined by flow cytometry (**A**). The HIV-1 gp120 binding is measured by the fluorescent bead gp120 binding assay on the flow cytometer. Fluorescent beads are indirectly coated with HIV-1 gp120 proteins (Geijtenbeek et al., 2000a) (**B**) and added to DC. After 30 min at 37 °C, the binding of fluorescent gp120 beads is measured by flow cytometry. The DC are gated based on forward-side-scatter pattern and the fraction of cells binding fluorescent beads is measured by flow cytometry (**C**). The specificity of the gp120 binding is determined by preincubating the DC with blocking antibodies against CD4 or DC-SIGN, and performing the gp120 binding assay. Blocking antibodies against DC-SIGN prevent gp120 binding and binding is mediated by the C-type lectin domain of DC-SIGN since both the polycarbohydrate mannan and the calcium-ion chelator EGTA inhibit the binding (**D**).

1997; Granelli-Piperno et al., 1996; Rubbert et al., 1998; Zaitseva et al., 1997) but immature DC, cultured from cord blood $CD34^+$ cells, were found to be positive for only CCR5 (Canque et al., 1996). Culturing of DC can influence their expression of chemokine receptors, and therefore in situ analysis is useful in determining the expression pattern of these HIV-1 coreceptors. These data suggest that HIV-1 coreceptor usage of cervical or vaginal DC is different from that observed in skin- and monocyte-derived DC, and that care must be taken in drawing conclusions regarding sexual transmission of HIV-1 from models using skin- or blood-derived DC. Similar striking results were obtained when DC in mucosal tissues, such as cervix, rectum and, uterus, were analyzed for the in situ expression of CD4 as well as CCR5: DC of the lamina propria expressed CD4 but lacked CCR5, strongly suggesting that DC, present at these sites of first contact with sexually transmitted HIV-1, do not mediate viral fusion and thus can not be infected by HIV-1.

Recently, we have identified the DC-specific HIV-1 *trans*-receptor DC-SIGN, through its high-affinity interaction with ICAM-3 (Geijtenbeek et al., 2000a). DC-SIGN is a C-type lectin that binds the HIV-1 gp120 protein. Monocyte-derived DC express high levels of DC-SIGN and intermediate levels of CD4 and CCR5 (Fig. 11.3*A*). HIV-1 gp120 binding to immature DC is completely mediated by DC-SIGN, and not by CD4/CCR5 as was measured by flow cytometry with the fluorescent bead gp120-binding assay (Fig. 11.3*B–D*). However, DC-SIGN does not function as a classical HIV-1 receptor inasmuch as it does not mediate HIV-1 entry (Curtis et al., 1992; Geijtenbeek et al., 2000a), as do CD4, CCR5, and CXCR4. We have demonstrated that DC-SIGN binds both M- and T-tropic HIV-1, but also promotes efficient infection *in trans* of cells that express CD4 and the appropriate coreceptors. The function of DC-SIGN as a *trans*-receptor is independent of the coexpression of CD4 and HIV-1 coreceptors. DC-SIGN is abundantly expressed on both monocyte- and $CD34^+$-derived DC, as well as in vivo dermal DC of the skin and mucosal tissues involved in sexual HIV-1 transmission (Geijtenbeek et al., 2000a). DC-SIGN is also specifically expressed on two DC precursors in blood that are $CD14^-$ and $CD14^+$ (Geijtenbeek et al., 2000c). DC-SIGN is abundantly expressed in vivo by DC present in the rectal, cervical, and uterine mucosa, and, in contrast, these DC lack CCR5. DC-SIGN may therefore play a crucial role in initial HIV-1 exposure by mediating HIV-1 binding to DC present in mucosal tissues, rather than infection of these cells.

DIRECT INFECTION OF DC WITH HIV-1?

Data regarding the capacity of DC to support viral replication are conflicting, ranging from resistance (Cameron et al., 1992a; Pinchuk et al., 1994; Weissman et al., 1995a) or nonproductive infection (Cameron et al., 1994; Granelli-Piperno et al., 1995; Tsunetsugu-Yokota et al., 1997) to productive infection with both M- and T-tropic strains (Blauvelt et al., 1997; Dittmar et al., 1997; Granelli-Piperno et al., 1995; Tsunetsugu-Yokota et al., 1997). Immature

monocyte-derived DC were productively infected at low levels by M-tropic HIV-1 in contrast to T-tropic HIV-1 (Delgado et al., 1998), even though both CCR5 and CXCR4 were expressed; upon maturation, the cells lost their ability to replicate HIV-1 but were still efficient in transmitting both M- and T-tropic viruses to activated T cells, demonstrating that replication is not necessary for HIV-1 transmission. The capacity to support HIV-1 replication depends on the maturation state of DC. Immature DC cultured from cord blood $CD34^+$ cells were permissive for M-tropic HIV-1 but not for T-tropic HIV-1, in accordance with the presence of CCR5 and the absence of CXCR4; after maturation, DC became permissive for T-tropic HIV-1 resulting from up regulation of CXCR4, although no productive infection occurred (Canque et al., 1996). Collectively, these experiments demonstrate that HIV-1 entry into DC can be detected but that it does not necessarily lead to a productive infection of these cells in the absence of T cells. The reasons for these discrepancies are still unclear, but they may reflect differences in DC isolation procedures and culture methods. The purity of the DC fractions used is essential, as low levels of contaminating T cells lead to severely compromised results regarding productive infection. Sensitivity of the detection method and the virus concentration used in the DC infection are also important variables. Moreover, HIV-1 susceptibility has been shown to be dependent on DC origin and maturation state (Canque et al., 1996; Delgado et al., 1998). Expression of CD4 differs greatly among various DC populations and expression of CCR5 and CXCR4 is strongly dependent on the maturation stage of DC.

Similar results were obtained with DC isolated from tissues. Skin-derived DC were not infected by HIV-1 in the absence of T cells, whereas a vigorous productive infection occurred when conjugates of DC with T cells were pulsed with HIV-1 (Ayehunie et al., 1995; Pope et al., 1994, 1995). Similarly, pulsing of isolated blood DC did not result in a productive infection (Cameron et al., 1992b; Weissman et al., 1995a). HIV-1-pulsed blood DC bound HIV-1 on their cell surface and efficiently enhanced infection of resting $CD4^+$ T cells (Weissman et al., 1995b).

Despite these conflicting reports, it is clear that DC are able, by the action of DC-SIGN, to capture HIV-1 at mucosal sites. DC might mediate viral entry to an extent during HIV-1 capture, depending on the expression of CD4, CCR5, and CXCR4, although this is a rather inefficient process compared with T cells. The observation that expression of CCR5 on DC is different between in vitro- and in vivo-derived DC (Geijtenbeek et al., 2000a) demonstrates that data obtained from HIV-1 infection of in vitro derived DC do not necessarily reflect the in vivo situation.

DC CAPTURE HIV-1 AND EFFICIENTLY TRANSMIT HIV-1 TO T CELLS

In contrast to the varying results obtained for the direct infection of DC with HIV-1, there is substantial evidence that all types of DC pulsed with HIV-1 in

vitro produce a vigorous infection when co-cultured with T cells (Ayehunie et al., 1995; Cameron et al., 1992b, 1994b; Pope et al., 1994; Weissman et al., 1995a). Early studies demonstrated that blood DC pulsed with HIV-1 are not infected themselves, but efficiently transferred virus to activated T cells. Co-culture of HIV-1-pulsed DC with activated T cells resulted in an efficient virus production, whereas without T cells no virus was produced. Experiments with different combinations of human and murine DC or T cells clearly demonstrated that both human and murine DC could transfer HIV-1 to T cells, but that human T cells were required for virus production, supporting the model that T cells are directly infected and that DC facilitate this process (Cameron et al., 1992b, 1994b). Similar experiments with human skin DC demonstrated that DC-T cell conjugates were sites of productive virus infection: viral entry into skin DC was demonstrated by the presence of *gag*-containing HIV-1 DNA, but a productive infection only occurred in the presence of T cells. Strikingly, a productive infection occurred even though the T cells were not activated by exogenous stimuli, indicating that the presence of DC was sufficient to activate T cells (Pope et al., 1994) and thus providing a suitable environment for HIV-1 replication.

A model has evolved based on these in vitro data in which DC play an essential role in HIV-1 capture and viral dissemination upon migration to the draining lymph nodes to enter the T-cell compartments. Studies of tonsils and adenoids from several HIV-1-infected patients identified many HIV-1-positive multinucleated syncytia within the T-cells areas, which contained DC (Frankel et al., 1996). These findings represent an in vivo correlation of the permissive DC–T-cell environment observed in in vitro experiments. Further evidence was provided by in vivo experiments using simian immunodeficiency virus (SIV)-1.

SIV-rhesus macaque models are being used to further advance our understanding of the interactions between DC and HIV-1, and to investigate the involvement of DC in HIV-1 dissemination, especially early after infection. One such model investigated the infection of tonsils in macaques (Stahl-Hennig et al., 1999): the stratified squamous epithelium overlying the tonsils is comparable to the lining of the vagina and anus, tissues implicated in genital transmission of HIV-1. Atraumatic application of pathogenic SIV-1 to macaque tonsils revealed that the infection started locally in the tonsils, but quickly spread to other lymphoid organs. At day 3, there were few infected cells, but the number increased rapidly, and reached a plateau after 4 days. The infection was not detected in the DC-rich squamous epithelium to which the virus was applied, but was found primarily in $CD4^+$ T cells in the T-cell area of tonsils, thus following the route of migrating DC. Similarly, during acute SIV-1 infection of macaques, virus replicated primarily in the T-cell areas of lymph nodes (Reimann et al., 1994). Possibly, DC had captured SIV-1 and carried it to the T-cell-rich areas for efficient infection, in accordance with another study in which rhesus macaques were intravaginally exposed to SIV-1 (Hu et al., 1999). SIV entered the vaginal mucosa within 60 min of exposure, infecting primarily intraepithelial DC, and SIV-infected cells were found in the draining lymph

nodes within 18 h. Similarly, the first target of SIV-1 administered intravaginally was identified as DC and lymphocytes, located in the vaginal submucosa (Spira et al., 1996). SIV-1 dissemination occurred in the draining lymph nodes of the genital tract, and virus was then systemically transported to other lymph nodes, following the migration pattern described for DC.

Therefore, DC are the initial targets for SIV-1 and transmit the virus to T cells in the lymphatic tissues. Discrepancies in the detection of DC infection can again be accounted for by different subtypes, sensitivity of detection, amount of HIV-1 used to infect, and time of analysis after infection. However, it is apparent that DC play an essential role in the dissemination of HIV-1, and the development of successful strategies to counter HIV-1 transmission demands a deeper understanding of the mechanisms by which DC act as HIV-1 carriers.

DC BIND, PROTECT, AND TRANSMIT HIV-1 TO T CELLS

Investigations into the role of DC in HIV-1 transmission have mainly focused on in vitro experiments and SIV-macaque in vivo models. It is becoming clear from these experiments that DC are indeed essential for HIV-1 dissemination after sexual transmission. Different DC populations are present in the various human mucosa involved in sexual HIV-1 transmission, and investigations regarding the HIV-1 receptor and co-receptor repertoire of these cells are important to understand their specific roles.

DC-SIGN is expressed by blood-borne DC, by both $CD34^+$ and monocyte-derived DC, and in vivo on dermal DC, on mature DC in lymphoid tissues and in mucosal tissues involved in sexual transmission of HIV-1 (Geijtenbeek et al., 2000a, b, c). DC-SIGN does not function as a classical HIV-1 receptor because it does not mediate HIV-1 entry. However, DC-SIGN binds both M- and T-tropic HIV-1 gp120 with high affinity and promotes efficient infection *in trans* of cells that express CD4 and the appropriate chemokine receptors. Moreover, this function of DC-SIGN as a *trans*-receptor is independent on the co-expression of CD4 and the HIV-1 co-receptors on DC (Geijtenbeek et al., 2000a).

Upon HIV-1 infiltration, immature DC migrate toward HIV-1 as has been shown in vitro (Lin et al., 2000). The high level of expression of DC-SIGN on immature DC, and its high affinity for gp120 (Fig. 11.3), indicate that DC-SIGN endows DC with the ability to efficiently capture HIV-1, even when the virus is present in minute amounts. HIV-1 may subsequently exploit the migratory capacity of DC to gain access to the T-cell areas of lymphoid tissues (Fig. 11.4). Time-course experiments have shown that DC-SIGN is able to capture and bind HIV-1 for more than 4 days, after which the virus is still able to infect permissive cells, allowing sufficient time for it to be transported by DC from mucosa to lymphoid compartments. Several groups have reported that DC can migrate from the periphery to draining lymph nodes within 2 days after antigen exposure or HIV-1 challenge (Barratt-Boyes et al., 1997; Stahl-Hennig

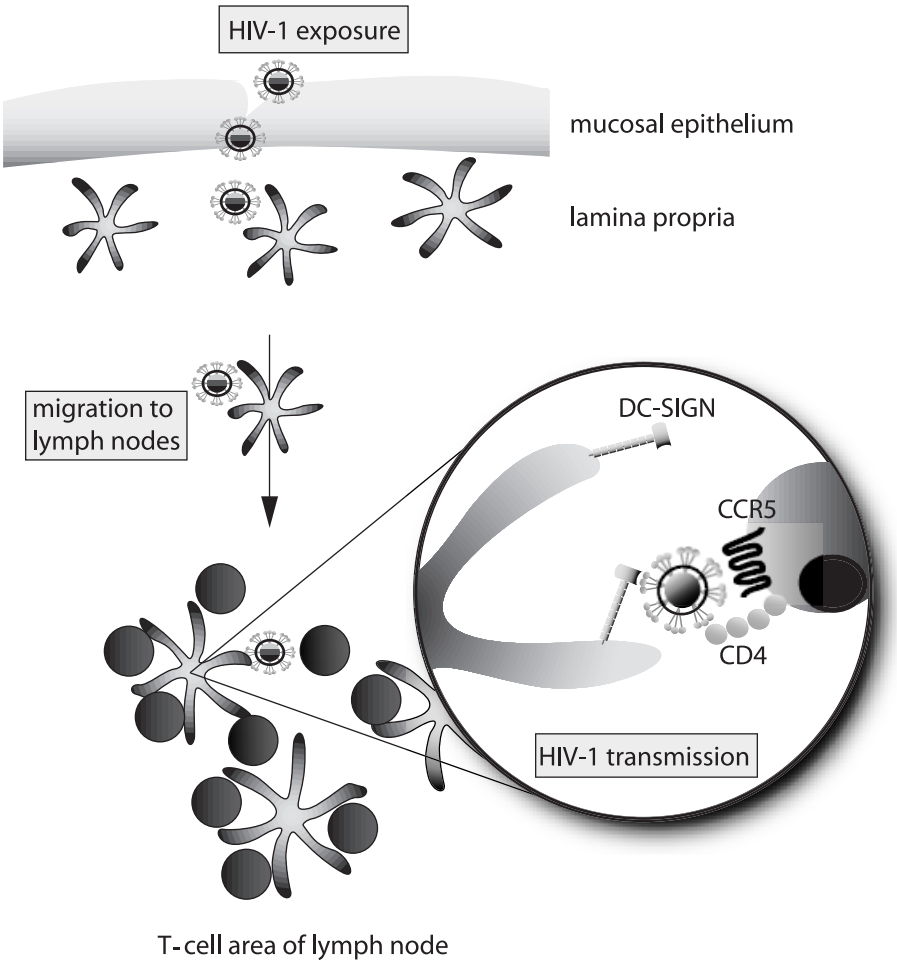

Figure 11.4. DC ferry HIV-1 from the peripheral tissues to the lymphoid compartments, where DC-bound HIV-1 infects $CD4^+$ T cells. DC are the first targets of HIV-1 after mucosal exposure and these cells are DC-SIGN positive. HIV-1 is bound by DC-SIGN, and the immature DC, carrying DC-SIGN-bound HIV-1, migrates to the lymphoid tissues where it clusters with T cells. DC-SIGN-bound HIV-1 is transmitted to $CD4^+$ T cells and DC-SIGN enhances the infection *in trans* leading to a productive and sustained infection.

et al., 1999). Viral particles have been reported within endocytic vesicles of DC, suggesting that DC-SIGN-bound HIV-1 may be internalized intact and protected during the time required for the cells to complete their journey to the draining lymph nodes. Our studies suggest that HIV-1 is then presented by DC, as particles bound by DC-SIGN, to the CD4/CCR5 complex present on T cells in the lymphoid compartment, and that this greatly enhances viral entry into these cells (Fig. 11.4). Blocking antibodies to DC-SIGN prevent both the ini-

tial capture of HIV-1 by DC-SIGN and the productive infection of T cells in a DC–T-cell co-culture. Therefore, even in the presence of the obligatory HIV-1 receptors on DC, DC-SIGN functions as a crucial *trans*-receptor for HIV-1 infection of T cells. Moreover, the *trans*-receptor function of DC-SIGN is independent of CD4 or HIV-1 co-receptor expression (Geijtenbeek et al., 2000a), explaining the finding that CCR5-negative DC from human genital tract enhanced the infection of T cells with M-tropic HIV-1 (Hladik et al., 1999).

CONCLUSION

As sentinels of the immune system, DC are exploited by HIV-1: DC capture HIV-1 and ferry the virus to T-cell-rich areas. Capture is mediated by DC-SIGN expressed on DC, and its function is distinct from that of CD4 and the HIV-1 coreceptors; DC-SIGN does not mediate HIV-1 entry into DC but enhances the HIV-1 infection of $CD4^+$ T cells *in trans*. It remains to be determined whether DC-SIGN has a significant role in HIV-1 pathogenesis in vivo, although our in vitro results and its expression in mucosal tissues are consistent with its having a key function in the early stages of viral infection after sexual transmission. However, a role in the persistence of T-cell infection can not be excluded as DC-$SIGN^+$ DC are located in the lymph nodes supporting a regulating role for DC-SIGN in chronic HIV-1 infection. The presence of DC-$SIGN^+$ DC precursors in blood (Geijtenbeek et al., 2000c) supports a further role for DC-SIGN-mediated HIV-1 transmission to T cells by HIV-1 infection through blood. DC isolated from blood have been shown to bind HIV-1 and efficiently transmit DC-bound HIV-1 to $CD4^+$ T cells (Cameron et al., 1992b; Weissman et al., 1995b). Future research will evaluate whether DC-SIGN is a potential target to inhibit initial HIV-1 infection, dissemination, and pathogenesis.

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PART III

PROCESSES

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12

Homeostasis and Restoration of the Immune System in HAART-Treated HIV-Infected Patients: Implication of Apoptosis

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INTRODUCTION

The primary receptor for human immunodeficiency virus (HIV) is the CD4 molecule, and the hallmark of HIV infection is the progressive destruction of the CD4 T lymphocyte subset and the development of severe immune deficiency. CD4 T lymphocytes represent key helper cells that contribute, through cytokine synthesis, to the differentiation of innate (including natural killer [NK] and γδ cells) and specific (cytotoxic CD8 T lymphocytes) antiviral cytotoxic cells. They also contribute, through chemokine synthesis, to the control of lymphocyte migration to the site of infection and to inhibition of HIV entry into CD4-expressing targets (Levy, 1998; Malkovsky et al., 2000).

After more than 15 years of investigation, the dynamic basis for CD4 T-cell depletion in acquired immunodeficiency syndrome (AIDS) is still a matter of debate. It was generally accepted that CD4 T-cell destruction is the direct consequence of HIV cytopathogenicity. However, the observation that a significant fraction of peripheral lymphocytes from asymptomatic HIV-infected patients

are prematuraly primed for activation-induced apoptosis has challenged the hypothesis that indirect virus-driven mechanisms are involved in the priming for apoptosis of non infected cells, leading to their destruction upon antigenic activation (Gougeon and Montagnier, 1993; Groux et al., 1992; Meyaard et al., 1992). The current understanding of CD4 T-cell homeostasis in the course of HIV infection is that the progressive depletion of CD4 T lymphocytes is the consequence of both their massive destruction by apoptosis triggered by the virus or its proteins and a deficient compensation by either peripheral expansion of mature lymphocytes or central production of new CD4 T cells.

Indeed, analysis in patients of the proportion of blood lymphocytes engaged in the cell cycle, either ex vivo by the detection of the nuclear molecule Ki67 or in vivo following injection of [^{2}H] glucose, has revealed that the CD4 T-cell turnover is two- to three-fold higher in HIV-infected patients compared with HIV-negative controls, and that production of new CD4 T cells in HIV-infected subjects is not significantly different from that in healthy controls (Fleury et al., 1998; Hellerstein et al., 1999; Sachsenberg et al., 1998). In fact, in addition to inducing apoptosis, virus replication inhibits CD4 T-cell production in primary lymphoid organs because, following suppression of HIV by highly active antiretroviral therapy (HAART), a dramatic increase in CD4 T-cell production is observed (Fleury et al., 1998, 2000), related to relative thymic activity and to the appearance in the blood of naive T cells expressing some products of the T-cell receptor rearrangement (TREC) characterizing recent thymic emigrants (Douek et al., 1998). Thus, the rapid increase in peripheral CD4 T cells in patients under HAART is the reflection of both the survival and regeneration of the CD4 T-cell pool. In the present chapter, we will discuss the molecular basis of HIV-dependent apoptosis, the consequences of exacerbated apoptosis on T-cell homeostasis, the restoration of immune functions under HAART, and the contribution of controlled apoptosis to this restoration.

APOPTOSIS IN HIV INFECTION

HIV Genes Trigger Apoptosis

In vitro infection of CD4 T lymphocytes with HIV is associated with a cytopathic effect of the virus, manifested by ballooning of cells and formation of syncytia, leading to the death by apoptosis of both infected and noninfected cells. Apoptosis is triggered by the viral envelope gp160, expressed on the surface of infected cells, which binds to accessible CD4 receptors on the surface of neighboring cells (Laurent-Crawford et al., 1991; Terai et al., 1991). Thus, chronically HIV-infected cells can serve as effector cells to induce apoptosis in uninfected target CD4 T cells. The apoptotic pathway involved in gp160-dependent apoptosis of uninfected CD4 T cells is not mediated by the CD95 or TNF-RI death molecules, but it involves caspases (Ohnimus et al., 1997). Several other HIV-1 gene products can influence the survival of infected cells

and bystander cells. Tat, a viral transcription factor, was found to up-regulate Bcl-2 expression, protecting cells from apoptosis (Zauli et al., 1995). In contrast, establishment of stable Tat-expressing cell lines or addition of exogenous Tat has been reported to sensitize cells to CD95-, T-cell receptor (TCR)-, or CD4-induced apoptosis (Li et al., 1995; Westendorp et al., 1995). The *vpr* gene, required for productive infection of nondividing cells, was shown to induce arrest of cells in the G2/M phase of the cell cycle, inducing apoptosis in human T cells and fibroblasts (Stewart et al., 1997; Yao et al., 1998). Another HIV gene, *vpu*, was reported to increase the susceptibility of infected peripheral T cells and Jurkatt T cells to CD95-induced apoptosis (Casella et al., 1999).

INAPPROPRIATE APOPTOSIS IN NONINFECTED LYMPHOCYTES FROM HIV+ PATIENTS

Peripheral blood T cells from asymptomatic HIV-infected persons are prematurely prone to activation-induced apoptosis, triggered ex vivo either by TCR ligation by specific antibodies or superantigens, by ligation of the death receptors CD95, tumor necrosis factor (TNF)-RI, and TNF-RII, or following activation by mitogens or phorbol myristate acetate (PMA)+ ionomycin (Fig. 12.1*B*). In addition, spontaneous apoptosis can be detected in patients' T cells following a short-term incubation in medium alone (Fig. 12.1*B*) (Gougeon et al., 1993; Groux et al., 1992; Meyaard et al., 1992). This premature cell death concerns not only CD4 T cells, but also cells that are not targets of HIV, such as CD8 T cells and B cells (Gougeon et al., 1996), and it can be detected in vivo in lymph nodes and tonsillar tissue of HIV-infected patients (Amendola et al., 1996; Muro-Cacho et al., 1995; Rosok et al., 1998). These facts, combined with the observation that apoptotic T cells in lymph node sections of HIV-infected children and SIV-infected macaques are dominant in uninfected bystander cells whereas infected cells are not apoptotic (Finkel et al., 1995), have prompted the hypothesis that CD4 T-cell depletion is not only the consequence of the cytopathic effect of HIV but also results from indirect mechanisms of apoptosis induction in noninfected cells.

Relation to Persistent Virus Expression and Disease Evolution

A general state of immune activation is observed in the asymptomatic phase of HIV infection both in lymphoid tissues and peripheral blood lymphocytes, and persists throughout the entire course of HIV infection. This is reflected by the expression of activation markers such as human leukocyte antigen (HLA)-DR, CD45R0, CD38 and CD95, in CD4 and CD8 T cells in lymph nodes and in peripheral blood, which increases with disease evolution (Fig. 12.1*A*) (Giorgi et al., 1993; Muro-Cacho et al., 1995). In addition to the unceasing expression of HIV antigens, exogenous factors such as opportunistic pathogens stimulate the production of proinflammatory cytokines, including TNF-α, interleukin (IL)-

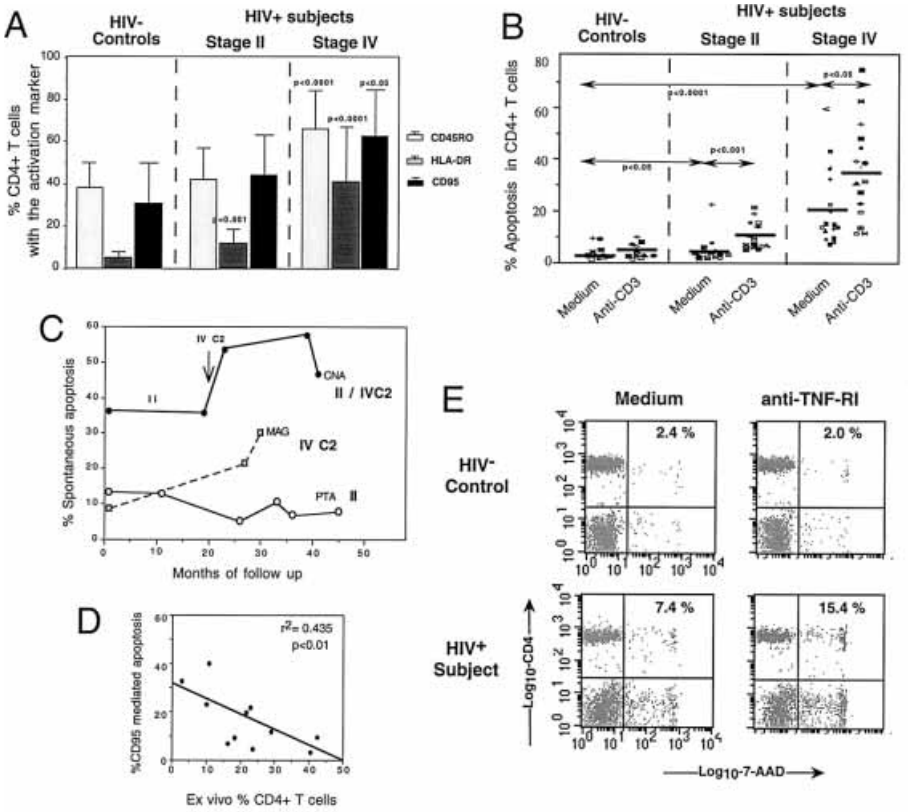

Figure 12.1. Inappropriate priming for apoptosis of peripheral lymphocytes from HIV-infected persons. PBMC from control donors or HIV^+ persons were analyzed ex vivo for the expression of activation markers (**A**) or the susceptibility for apoptosis induced by overnight incubation in medium (spontaneous) (**B**, **C**), anti-CD3 monoclonal antibodies (mAbs) (**B**), anti-CD95 mAbs (**B**) or anti-TNF-RI mAbs (**E**). (**A**) The expression of the indicated activation markers has been analyzed by flow cytometry on PBMC from controls, stage II or stage IV untreated HIV-infected persons, and the data represent the mean percentage of CD4 T cells expressing the corresponding activation marker within total CD4 T cells. *p* Values indicate significant differences compared with control donors. (**B**) PBMC from the same subjects have been cultured overnight in medium or having been coated with anti-CD3 mAbs. Apoptosis was measured using the 7-AAD staining, as described (Gougeon et al., 1996). The mean percentage of apoptotic cells within the CD4 T-cell subset is shown. (**C**) Spontaneous apoptosis (overnight incubation in medium) within the CD4 subset has been followed in three patients over 4 years. The clinical stages of the patients are indicated and for patients CNA, the arrow shows the progression from stage II to stage IV C2. (**D**) Anti-CD95-induced apoptosis has been measured in PBMC from a series of nontreated patients, and the correlation between the percentage of apoptosis and disease evolution (ex vivo percentage of CD4 T cells) is shown. (**E**) FACS dot plots representative of the analysis of TNF-RI-induced apoptosis in PBMC from a control donor and an HIV-infected patient. Apoptosis in CD4 T cells is measured by incorporation of 7-AAD into the nucleus. TNF-RI ligation induces apoptotic cell death in T cells from the patient although it has no effect in those from the control donor.

1β, and IL-6, which drive cellular activation and viral replication (Blanchard et al., 1997). This unbalanced immune activation might be the primary mechanism responsible for premature lymphocyte death in AIDS. Indeed, apoptotic cells in patients' lymphoid tissues and in blood exhibit an activated phenotype (Gougeon et al., 1996; Muro-Cacho et al., 1995) and there is a statistically significant correlation between the intensity of spontaneous, TCR-triggered apoptosis in both CD4 and CD8 subsets and their in vivo activation state (Gougeon et al., 1996). In addition, we have found that the nonpathogenic HIV-1 infection in chimpanzees and the lower pathogenicity of HIV-2 infection compared with HIV-1 in African patients are associated with a lack of immune activation and very low level of T-cell apoptosis (Gougeon et al., 1997; Michel et al., 2000). Therefore, the physiological role played by apoptosis in the homeostatic control of cell numbers following primary infection, normally ensuring the clearance of activated lymphocytes in order to terminate the immune response, might be detrimental for the immune system in the case of a chronic infection with persistent viral antigen release, such as that induced by HIV.

The relevance of programmed cell death (PCD) to AIDS pathogenesis has been suggested by several studies. For example, the proportion of CD4 and CD8 T lymphocytes undergoing apoptosis spontaneously or after ligation of the TCR or the CD95 receptor increases with disease evolution, evaluated by the in vivo reduction of the CD4 T-cell number (Fig. 12.1, *C* and *D*) (Böhler et al., 1997; Gougeon et al., 1996; Sloand et al., 1997). In addition, comparative studies in pathogenic models of lentiviral infection, including macaques infected with simian immunodeficiency virus (SIV) (Estaquier et al., 1994; Gougeon et al., 1993), vs. nonpathogenic models including SIV-infected african green monkeys (Estaquier et al., 1994) or chimpanzees infected with HIV or SIVcpz (Gougeon et al., 1997), revealed that increased lymphocyte apoptosis is only observed in pathogenic lentiviral infections. This suggests that apoptosis can significantly contribute to AIDS pathogenesis and it could be the mechanism responsible for the clearance of activated but healthy T cells and consequently contribute to the impoverishment of the pool of effectors (Th and CTL) and antigen-presenting cells (Gougeon, 1999).

Role of Death Receptors

At the molecular level, the unbalanced immune activation in HIV-1 infection is responsible for the in vivo down-regulation of Bcl-2 and up-regulation of CD95/CD95L expression in patients' lymphocytes and monocytes. Indeed, we have shown that, in vivo, a significant fraction of peripheral T cells of patients exhibit a low level of Bcl-2, which primes them for spontaneous apoptosis after a short-term culture (Boudet et al., 1996). This population is expanded with disease evolution and it is not found in the nonpathogenic model of HIV infection in chimpanzees (Gougeon et al., 1997). Phenotypic analysis of this low Bcl-2 T-cell subset indicated that it mostly includes activated CD8 T cells, lacking CD28 expression and expressing the cytotoxic TIA-1 granules, sug-

gesting that they are cytotoxic cells (Boudet et al., 1996). Interestingly, a similar subset has been described in vivo in patients' lymph nodes, with comparable phenotype, i.e., $CD8^+CD45R0^+TIA\text{-}1^+$ Bcl-2 (low) (Bofill et al., 1995).

The lack, in vivo, of survival factors, such as IL-2 or IL-15, known to up-regulate Bcl-2 expression and promote cell survival, might be responsible for the priming for apoptosis of CD8 cells with cytotoxic characteristics, and may contribute to the loss of antiviral immunity throughout HIV infection. IL-2 can rescue cells from spontaneous apoptosis induced by growth factor withdrawal, and this rescue is controlled by molecules from the Bcl-2 family (Akbar et al., 1996). Administration of IL-2 to HIV-infected individuals has been shown to significantly increase CD4 T cell numbers and preserve antiviral immune functions (Gougeon et al., 2000; Levy et al., 1999). IL-15 exhibits many activities in common with IL-2, attributable to the utilization by both cytokines of two common receptor chains, β and γ, and recent attention has turned toward IL-15 as a possible alternative immunotherapy. Comparison of the biological effects of IL-2 and IL-15 on lymphocytes from HIV-infected patients indicated that IL-15 had a more potent survival effect on NK cells as well as on naive CD4 T cells, which are preferentially lost in HIV-infected individuals (Naora and Gougeon, 1999a, b). In addition, IL-15 was found to act as a potent survival factor in the prevention of spontaneous apoptosis, up-regulating Bcl-2 expression and stimulating lymphocyte proliferation (Naora and Gougeon, 1999c). Therefore, lymphocyte loss occurring by growth factor deprivation in HIV infection may be potentially prevented by IL-2 or IL-15.

Death receptors belonging to the TNF-R superfamily are involved in HIV-dependent apoptosis. The CD95 receptor is highly expressed on both CD4 and CD8 T cells from patients, which show an increased susceptibility to CD95-induced apoptosis, positively correlated with disease progression (Fig. 12.1*D*) (Boudet et al., 1996; Debatin et al., 1994; Gougeon et al., 1999; Katsikis et al., 1995). CD95L is also up-regulated in patients' lymphocytes (Mitra et al., 1996) and in macrophages from lymphoid tissues, and its expression is correlated with the degree of tissue apoptosis (Badley et al., 1996; Dockrell et al., 1998). CD95L up-regulation on CD4 T cells and macrophages can be induced in vitro by HIV infection or through the direct effect of viral proteins such as gp120, Tat, or Nef (Mitra et al., 1996; Westendorp et al., 1995), which makes them possible effectors in killing CD95-expressing cells. Indeed, we have shown that a CTL clone, derived from a patient's peripheral T cells, specific for a Nef peptide in the context of HLA class I molecule, is able to mediate both perforin- and Fas-mediated dependent cytotoxic activities on Nef-presenting target cells and CD95-expressing compliant cells, respectively (Garcia et al., 1997). Moreover, CD4 T cells expressing CD95L can kill CD8 T cells via CD95/CD95L-mediated apoptosis (Piazza et al., 1997). Thus, the CD95 system is dysregulated in HIV infection and the biological relevance of these observations in the context of the chronic and active HIV infection may be discussed. The high plasma viral load associated with active HIV replication in lymphoid organs triggers a strong antigenic activation, hence exacerbating the CD95/

CD95L system and promoting nonprofessional CD95L-expressing killer cells, which may be responsible for the destruction of CD95-expressing cells, abundant in HIV-infected patients. Interestingly, experiments performed on lymphocytes from HIV-infected chimpanzees revealed that their resistance to CD4 T-cell depletion is associated with the lack of susceptibility of their T lymphocytes to CD95-induced apoptosis (Gougeon et al., 1997).

In addition to CD95L, other members of the TNF family are involved in HIV-induced apoptotic cell death. We have recently shown that both CD4 and CD8 T cells from HIV-infected persons are highly susceptible to TNF-RI and also TNF-RII-mediated apoptosis (Fig. 12.1*F*), and the molecular basis of this process are currently under investigation. Apoptosis in CD8 T cells may involve the TNF/TNF-R system inasmuch as in vitro binding of HIV gp120 on the chemokine receptor CXCR4, coreceptor for HIV, induces both the up-regulation of membrane TNF on macrophages and TNF-RII on peripheral CD8 T cells, leading to macrophage-dependent apoptosis of CD8 T cells (Herbein et al., 1998). The binding of HIV gp120 on CXCR4 also induces a rapid cell death in normal CD4 T lymphocytes, independent of CD95, and inhibited by SDF-1 (Berndt et al., 1998). Finally, TRAIL (TNF-related-apoptosis-inducing-ligand) was identified as an apoptotic inducing factor on T cells from HIV-infected patients, not on normal T cells (Katsikis et al., 1997).

Impact on Cytokine Synthesis

Upon antigenic activation, naive T cells proliferate and, according to the cytokine environment and the nature of the costimulatory signals delivered by the antigen-presenting cells, they can differentiate into effector cells with polarized patterns of cytokine synthesis. Proinflammatory Th1 cells secrete IL-2, interferon (IFN)-γ, and TNF-α, which are important mediators of cellular immunity and are involved in activation of macrophages, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, which provide help to B cells for immunoglobulin (Ig) synthesis, mediate eosinophilia, and antagonize the macrophage-activating action of Th1 cytokines. HIV-infection induces an early impairment of cytokine synthesis because it is accompanied by a decline of in vitro production of IL-2, IL-12, and IFN-γ by peripheral blood mononuclear cells (PBMCs) in response to recall antigens, an increased production of IL-4, IL-6, and IL-10, and this Th1$\rightarrow$Th2 shift is predictive for the reduction in CD4 cell counts, time to AIDS diagnosis, and time to death (Clerici and Shearer, 1994). To study the possible impact of apoptosis on alteration in cytokine synthesis, we have developed a single-cell analysis method that allows enumeration of Th1/Th2 subsets derived from polyclonally short-term stimulated peripheral T cells and concomittant analysis of their priming for apoptosis (Lecoeur et al., 1998). With that approach and under the experimental conditions used (16-h stimulation of PBMC with PMA + ionomycin), we found that HIV infection is associated with a differential alteration in the representation of Th1 subsets, rather than a commitment of T cells to secrete Th2 cytokines (Ledru et al.,

1998). Indeed, compared with healthy donors, a significant decrease in the frequency of T cells primed for IL-2 or TNF-α was observed, whereas that of T cells primed for IFN-γ was unchanged. The disappearance of IL-2-producing T cells in HIV-infected persons appeared to be a good indicator of disease progression and to correlate with the progressive shrinkage of the naive $CD45RA^{+}CD4^{+}$ T-cell compartment, the main producer of IL-2 (Ledru et al., 1998).

Analysis of the priming for apoptosis of T-helper-cell subsets revealed that the intrinsic capacity of lymphocytes to produce a given cytokine can influence their survival (Ledru et al., 1998). Indeed, T lymphocytes committed to IFN-γ or TNF-α production are more sensitive to activation-induced apoptosis than lymphocytes committed to IL-2 production. This gradient of susceptibility to apoptosis (IL-2 < IFN-γ < TNF-α) was detected in both CD4 and CD8 T-cell subsets, both in control donors and HIV-infected patients, in whom the susceptibility to apoptosis of IL-2 and TNF-α producers was increased compared with controls. In addition, this differential intrinsic susceptibility to apoptosis of Th1 effectors is tightly regulated by Bcl-2 expression. In HIV-infected persons, the progressive decrease in the proportion of IL-2-producing T cells is correlated with their susceptibility to apoptosis and disease progression. On the other hand, the frequency of IFN-γ producers is preserved in patients despite an increased rate of apoptosis in this subset, which is counterbalanced by an increased proportion of $CD45R0^{+}$ CD8 T cells', the main IFN-γ producers, survival (Ledru et al., 1998). Altogether, these observations indicate the important relationship between increased priming for apoptosis in peripheral T cells, alteration in cytokine synthesis, and disease evolution.

IMPACT OF HAART ON T-CELL HOMEOSTASIS AND IMMUNE RESTORATION

HAART Regulates Lymphocyte Apoptosis

Antiviral therapy for HIV-infected patients has greatly improved in the recent years, and administration of HAART, combining HIV reverse transcriptase (RTIs) and protease inhibitors (PIs), has been successful in decreasing plasma viremia to undetectable levels, and dramatically changed the clinical course in a substantial proportion of patients (Flexner, 1998). However, the use of these combinations for prolonged periods of time is not without drawbacks, such as adherence to complex drugs regimen, risks of side effects, and viral resistance. Furthermore, different groups demonstrated that the pool of latently infected cells persists in essentially all infected patients tested who were receiving HAART for a long period of time (up to 3 years) and in whom suppression of plasma viremia has been maintained below the levels of detection by the most sensitive assays (Finzi et al., 1999, Zhang et al., 1999).

These potent therapies have challenged the question of the mechanisms

controlling homeostasis and reconstitution of the immune system in treated patients, and recent studies have pointed out the complexity of the mechanisms involved. The increase in CD4 T cells after HAART results from a combination of release of sequestered cells from lymphoid compartments to peripheral blood (Pakker et al., 1998), increased production rate of circulating T lymphocytes due to peripheral T-cell expansion (Fleury et al., 1998; Hellerstein et al., 1999), suppression of apoptosis (Gougeon et al., 1999) and central production of new T cells (Douek et al., 1998), suggesting that the mechanisms of renewal of CD4 T cells are still operational in chronically infected patients. Rapidly after initiation of HAART, an important drop in spontaneous and activation- and CD95-triggered apoptosis is observed in both CD4 and CD8 T cells from treated patients. This generally occurs before the decrease in the expression of activation markers CD45R0, HLA-DR, and CD95, but 6 months post-therapy both T-cell apoptosis and immune activation are suppressed, reaching control values (Gougeon et al., 1999; Sloand et al., 1999). This is shown in three representative patients in Figure 12.2*A*. In addition, suppression of the intrinsic fragility of patients' T cells under antiretroviral therapy is associated with normalization of the survival rate of activated lymphocytes, as shown in Figure 12.2*B* in an 18-month longitudinal study of three patients under HAART. Thus, potent inhibition of HIV replication is associated with normalization of lymphocyte apoptosis, and it is probably a multifactorial process. Indeed, mechanisms involved in apoptosis susceptibility of patients' lymphocytes during the chronic phase of HIV infection, i.e., in vivo immune activation and proapoptotic effect of some HIV proteins, are downregulated under HAART, restoring the survival of patients' lymphocytes. but we cannot exclude a direct in vivo immunomodulatory effect on T cells of anti-HIV drugs, including PIs, some of them being recently shown to suppress in vitro physiological apoptosis by inhibition of cellular proteases (Sloand et al., 1999). However, the inhibitory effect of HAART on ex vivo CD95- or anti-TNF-R-induced apoptosis is not observed in all the patients, in spite of good immunological (increased CD4 count) and virological (suppressed viral load) responses. Indeed, we have recently observed that certain antiretroviral drug regimens do not control both ex vivo spontaneous and activation-induced T-cell apoptosis, and, although it is not related to the viral load, it is correlated with the CD4/CD8 ratio and the type of RTI used (L. de Oliveira Pinto et al., unpublished data).

Partial Restoration of IL-2 Synthesis and Alteration of TNF-α T-cell Homeostasis Under HAART

As discussed above, we have shown that chronic HIV infection is associated with an altered pattern of Th1 subsets, characterized by a progressive decrease in the number of IL-2 and TNF-α producers, which was correlated with their increased priming for apoptosis. Because the level of lymphocyte apoptosis is normalized under HAART, we asked whether these potent therapies could

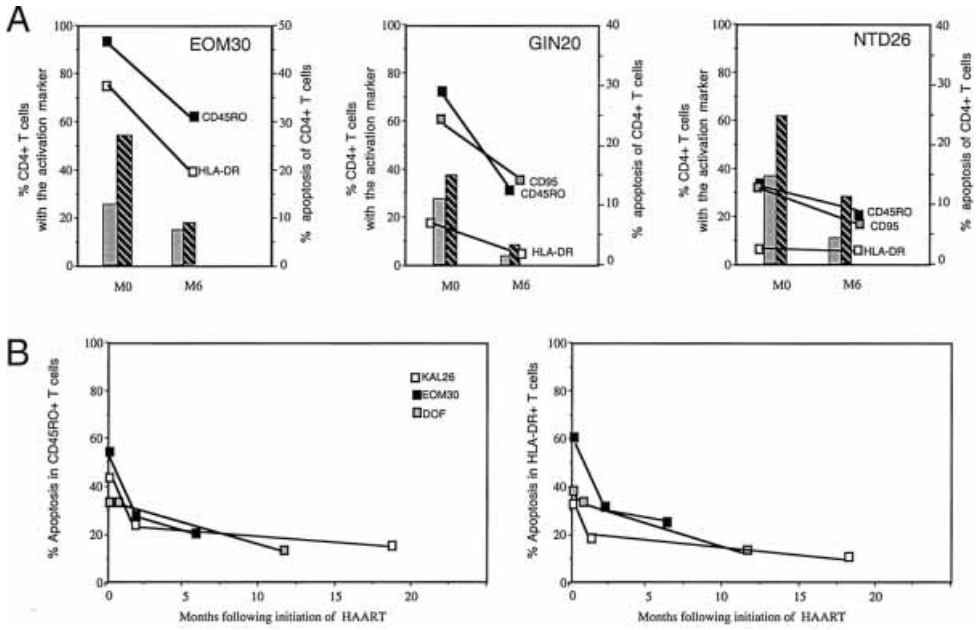

Figure 12.2. Influence of HAART on the expression of activation markers and priming for apoptosis in peripheral lymphocytes from HIV-infected persons. (**A**) Three representative patients have been followed for the ex vivo expression of the activation markers (*lines*) and spontaneous (*gray bars*) or anti-CD3-induced apoptosis (*hatched bars*) in CD4 T cells at initiation (*M0*) or after 6 months (*M6*) of HAART treatment. HAART induces a strong decrease of both activation markers and spontaneous and activation-induced apoptosis. (**B**) 18 months follow-up of the susceptibility to spontaneous apoptosis of memory ($CD45R0^+$) and activated ($HLA\text{-}DR^+$) lymphocytes from three representative patients. In addition to decrease the expression of activation markers, HAART induces a relative normalization of the intrinsic fragility of activated lymphocytes.

restore cytokine synthesis. This was done at the single-cell level by the intracellular detection by fluorescence-activated cell sorter (FACS) analysis of cytokines produced by CD4 and CD8 T cells in response to the polyclonal activation PMA + ionomycin. Concerning the restoration of IL-2 synthesis, it appeared that HAART induces a progressive increase in the percentage and absolute number of IL-2-producing CD3 T cells, as illustrated on the dot plots shown in Figure 12.3*A*. However, only an incomplete restoration of IL-2 synthesis was observed after 18 months of HAART, and analysis of IL-2 production by naive and memory T-cell subsets revealed that this was due to the altered capacity of naive CD4 T cells from these patients to produce IL-2 (E. Ledru et al., unpublished data). These observations raise the question of the functionality of the naive CD4 subset that emerges under HAART. Concerning IFN-γ synthesis, the normal proportions of IFN-γ producers before treatment slightly decreased after HAART (Fig. 12.3*A*). In contrast, we found that HAART is associated with an altered homeostasis of TNF-α producers. Indeed a progres-

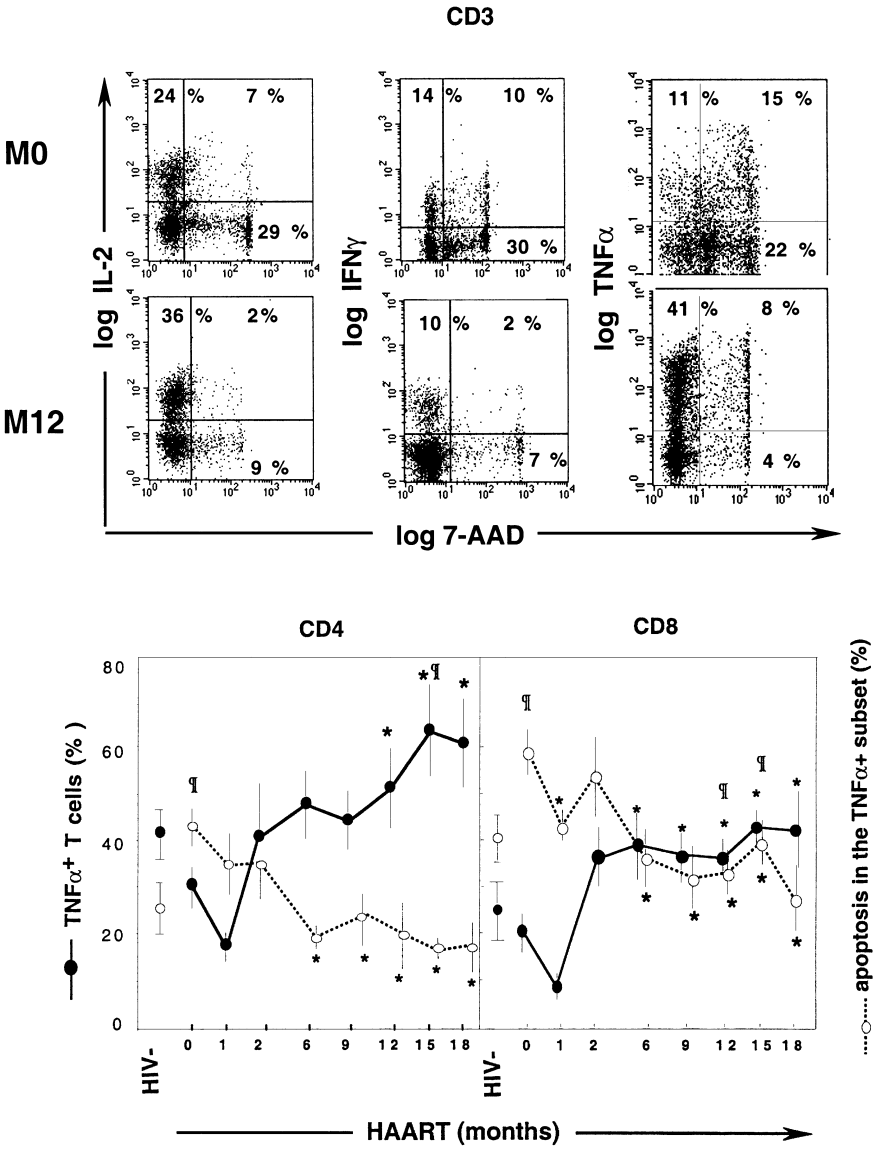

Figure 12.3. Influence of HAART on the priming of T cells for Th1 cytokines. (**A**) Intracellular cytokine analysis by FACS of PBMC stimulated overnight with PMA + ionomycin. PBMC from a representative patient have been analyzed for IL-2, IFN-γ, and TNF-α production at initiation of the treatment (*M0*) and after 1 year (*M12*). Concomitant analysis of intracellular cytokine synthesis, apoptosis, and T-cell marker CD3 has been performed as described (Ledru et al., 1998). It is shown that, under HAART, there is an important decrease of activation-induced apoptosis within cytokine producers, and this is particularly observed for TNF-α producers. In this patient, the proportion of IL-2 producers is slightly increased under HAART, whereas the proportion of IFN-γ producers is decreased. (**B**) Concomitant increase in the percentage of CD4 and CD8 T cells producing TNF-α during an 18-month period of HAART, and decrease of apoptosis within the same subset. Data represent the mean values from 15 patients (Ledru et al., 2000). * $p < 0.005$ compared with baseline value at M0. ¶ $p < 0.05$ compared with control HIV$^-$ donors.

sive accumulation of both CD4 and CD8 T cells primed for TNF-α synthesis is detected in the blood of treated patients (Fig. 12.3*A*) (Ledru et al., 2000). Because apoptosis plays an essential role in the negative regulation of TNF-α synthesis by T cells (Ledru et al., 1998), and apoptosis is suppressed under HAART, we combined the detection of apoptosis with that of cytokine synthesis on patients' T cells in order to analyze the relationship between both processes. As shown in Figure 12.3*B*, the accumulation of TNF-α T-cell producers under HAART is correlated with the suppression of physiological apoptosis in this subset. This is partly related to the cosynthesis by TNF-α T-cell producers of IL-2, an antiapoptotic factor (Ledru et al., 2000). In addition, the possible in vivo antiapoptotic influence of PI, by a direct effect on cellular proteases (Sloand et al., 1999), may contribute to the accumulation of TNF-α T-cell producers. Therefore, the re-emergence of CD4 T cells consequent to neutralization of HIV by HAART is not associated with the complete restoration of cytokine synthesis. Moreover, recent studies have shown that HIV-specific CD4 and CD8 responses, which may contribute to the containment of HIV replication (Ortiz, 1999; Schmitz, 1999) decline during HAART and become undetectable (Pitcher et al., 1998). More studies are needed to evaluate the renewal capacities of the immune system when HIV replication is controlled.

HAART-Associated Lipodystrophy Syndrome: Relation with Hormonal and Cytokine Dysfunctions

Long-term HAART has been associated with a unique and unexpected syndrome consisting of alterations in lipid metabolism and body fat redistribution, also called lipodystrophy. Lipodystrophy (LD) is associated with hyperlipidemia, insulin resistance, peripheral loss of fatty tissue, and visceral abdominal fat accumulation (Carr et al., 1998). The pathogenesis of this syndrome, which may lead to premature coronary artery disease as recently reported in PI-treated HIV-infected patients, remains unknown. Because important hormonal alterations, such as increased androgen and cortisol levels, were reported in chronically HIV-infected patients (Christeff et al., 1997) and considering that lipid metabolism is under the control of glucocorticoids, androgens, and cytokines, we asked whether peripheral LD is related to hormonal and immunological dysfunctions. We found that increased atherogenic lipids associated with LD are also associated with increased cortisol, decreased DHEA serum levels, and consequently increased cortisol/DHEA ratio in HAART-treated LD^+ vs. LD^- patients (Christeff et al., 1999). In LD^+ patients, elevated cortisol will stimulate peripheral adipocyte lipolysis. In parallel, because of the direct effect of DHEA on lipoprotein lipase activity and on peripheral insulin resistance, the decrease in DHEA in LD^+ patients may contribute to the reduction in peripheral lipogenesis. Thus, the combined DHEA decrease and elevated cortisol in LD^+ patients is probably responsible for the imbalance between lypolysis and lipogenesis in the peripheral adipose tissues.

Cytokines, such as TNF-α, induce a number of alterations in lipid metabo-

lism that can produce hyperlipidaemia. Indeed, TNF-α inhibits the intake of free fatty acids by adipocytes via the inhibition of lipoprotein lipase, leading to fat wasting, but also increases lipogenesis via the stimulation of the hepatic triglyceride synthetase, leading to hyperlipidaemia (Grunfeld et al., 1990). In addition, TNF-α promotes insulin resistance via both the increase in circulating free fatty acids and a direct action on the insulin receptor. Interestingly, the T-cell polarization to TNF-α synthesis that we observed in HAART-treated patients seems to favor the development of the lipodystrophy syndrome by contributing to alteration in lipid metabolism. Indeed, we found significant positive correlations between the absolute number of TNF-α CD8 T-cell precursors and lipid parameters usually altered in lipodystrophy including cholesterol, triglycerides, and the atherogenic ratio apolipoprotein B(Apo B)/Apo A1 (Ledru et al., 2000). Thus, new potent multitherapies can induce metabolic complications, which are related to hormonal and cytokine dysfunctions. In addition, recent studies have reported unusual clinical inflammatory syndromes after initiation of HAART, such as immune recovery vitritis (IRV) in cytomegalovirus retinitis patients or focal mycobacterial lymphadenitis. Our observations suggest that, through the dysregulation of TNF-α T-cell homeostasis, HAART creates a proinflammatory environment that might contribute to the development of these immune restoration inflammatory diseases.

CONCLUSION

The main role of apoptosis in specific immunity is to limit the antigen-specific clonal expansion, thus contributing to the termination of the response and prevention of autoimmunity. Premature lymphocyte apoptosis in the context of HIV infection is the consequence of continuous production of viral proteins, leading to an unbalanced immune activation and to the triggering of apoptotic programs in all lymphocyte subsets, and turning nonprofessional T cells into cytotoxic cells able to kill healthy noninfected cells through the CD95 pathway. Premature apoptosis is also responsible for the disappearance of T-helper cells primed for type-1 cytokine synthesis, thus contributing to the lack of cytokines/survival factors essential to the maintenance of HIV-specific immune response. Under potent antiretroviral therapies, a significant decrease in spontaneous or TCR- or CD95-induced lymphocyte apoptosis is observed, concomitant with a partial quantitative and qualitative restoration of the immune system in treated patients. However, owing to the suppression of physiological apoptosis, possibly because of antiapoptotic effects of some of the anti-HIV drugs, potent therapies are associated with alteration of TNF-α T-cell homeostasis, leading to an accumulation in the blood of T cells primed for TNF-α synthesis, and contributing to the development of metabolic complications such as hyperlipidemia and insulin resistance, associated with peripheral loss of fatty tissue characteristic of the lipodystrophy syndrome, as recently identified in patients with HAART.

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Mitochondria Functionality During HIV Infection

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INTRODUCTION: THE DUAL ROLE OF MITOCHONDRIA IN CELL LIFE AND DEATH

It is well established that at least two different main types of cell death exist, i.e., necrosis and apoptosis, with peculiar characteristics. The former is the outcome of a degenerative process or the passive consequence of gross injury to the cell; the latter represents the end point of a process that actively involves several cellular structures and organelles, including mitochondria. Apoptosis is a crucial defense mechanism that has evolved to favor the elimination of cells that are damaged or mutated. Classical studies have clarified that this type of death is first decided, then driven by the cell in a finely tuned fashion. Apoptosis has a pivotal importance in all multicellular living organisms, being involved in several physiological and pathological phenomena, including, among others, embryogenesis, differentiation, development of the immune system, control of cell growth, oncogenesis, and viral production (Kerr et al., 1972; Laurent-Crawford et al., 1991; Lee et al., 1993; Sen and D'Incalci, 1992; Vaux et al.,

1994; Wyllie et al., 1980). Apoptosis can be triggered by a wide variety of stimuli, such as deprivation of growth factors, γ-irradiation, oxygen free radical (OFR) production, receptor-ligand interaction, and inhibition of protein kinases. Each stimulus activates its own ''private'' pathway, which is thought to converge into one or a few central mechanism, the ''common pathway,'' leading to the classical morphological and biochemical changes found in the cell dying through apoptosis (Golstein, 1997; Hetts, 1998).

The phenomenon of apoptosis has received unprecedented attention in the past decade. Recently, there has been persistent and growing interest in the role of mitochondria during the apoptotic phenomena that occur in several physiological and pathological conditions, including infection by HIV. Initially, the focus was on the possible role of these organelles in supplying the energy (in the form of adenosine triphoshate [ATP]) required for such processes to occur (Richter et al., 1996). In the mitochondrial respiratory chain, the energy released by oxidation reactions is stored as an electrochemical gradient generated by the active extrusion of protons from the mitochondrial matrix to the cytosol. Such a gradient consists of two components: a transmembrane electrical potential of about 180–200 mV, negative inside, and a proton gradient of about 1 pH unit. This energy drives the synthesis of ATP, which is exported into the cytoplasm and then used, so that mitochondria are, in fact, the main power stations for aerobic life.

Only recently have scientists understood that these organelles are able to play two completely opposite roles, determining life or death for the cell. Not only are they of crucial importance for the generation of intracellular energy, but they are also capable of generating or amplifying signals that ultimately lead to programmed cell death/apoptosis. According to a very recent view, it seems likely that mitochondria possess an autonomic system that allows them to be degraded when irreversibly damaged, thus committing a sort of suicide, called ''mitoptosis'' by analogy with apoptosis (Skulachev, 1998, 2000). Massive mitoptosis resulting from a massive stimulus such as, for example, that provided by large amounts of reactive oxygen species (ROS), which, however, are mostly produced in mitochondria (Skulachev, 1996), can result in apoptosis because of the release of apoptogenic proteins such as cytochrome c (cyt c) or the apoptosis-inducing factor (AIF) that are normally present within the organelle.

Even before the identification of cyt c and AIF, a crucial role for this organelle during apoptosis was postulated because of the observation that the antiapoptotic protein Bcl-2 is present in the outer mitochondrial membrane (de Jong et al., 1994; Monaghan et al., 1992). Indeed, it was found that either Bcl-2 protein or other members of the same family are preferentially located at the contact sites where the inner and outer membranes come into close apposition (de Jong et al., 1994), near to proteaceous structures that form pores in the inner mitochondrial membrane. Such pores are referred to as mitochondrial ''megachannels''or permeability transition pores (Zoratti and Szabò, 1995). The opening of these pores allows for the free distribution of solutes $<$1500 Da and

is thought to cause decrease of the mitochondrial membrane potential ($\Delta\psi$) (Zamzami et al., 1995, 1996; Zoratti and Szabò, 1995), swelling of the organelle, rupture of the outer membrane, and release of cyt c (Liu et al., 1996) and/or AIF (Susin et al., 1996, 1999) from the intermembrane space. Both the latter compounds are able to induce nuclear apoptosis, i.e., apoptotic changes in isolated nuclei, that can be inhibited by Bcl-2 protein activity (Susin et al., 1996) and by protease inhibitors (Marchetti et al., 1996). AIF seems to activate apoptosis directly without the activation of caspases (reviewed in (Daugas et al., 2000)), whereas cyt c activates caspases through its effects on Apaf-1 cytosolic factor (Zhou et al., 1997). Upon binding of cyt c, Apaf-1 becomes competent at binding and activates procaspase-9 (Li et al., 1997; Zhou et al., 1997). The antiapoptotic or proapoptotic effect of Bcl-2 family members may reside in the regulation of the release of such apoptogenic molecules into cytosol. However, the precise mechanism by which they promote or prevent cell death is far from clear.

On the whole, it is evident that mitochondria are deeply involved in cell death, and it has been proposed that they might act as the central executioner of apoptosis (Susin et al., 1997). It has also been suggested that the fall in $\Delta\psi$ could be a common feature of the apoptotic program. Several groups investigating this point have produced conflicting (or contradictory) results, but these were likely due, at least in part, to different experimental models, or to the interpretation of data obtained using different dyes. In fact, $\Delta\psi$ has been measured by flow cytometry or confocal microscopy using a variety of fluorescent probes with different sensitivities to this parameter (Lopez-Mediavilla et al., 1989; Salvioli et al., 1997). In any case, it has been demonstrated that the release of cyt c can occur without a simultaneous drop in $\Delta\psi$ (Kluck et al., 1997; Yang et al., 1997). It is not clear how a protein like cyt c can transit from the intermembrane space into the cytosol without disruption of $\Delta\psi$. It has been proposed that pores are created ex novo in the outer membrane, allowing some cyt c to escape, while leaving the permeability of the inner membrane intact (Vander Heiden, et al., 1997). Therefore, it has been postulated that the release of apoptogenic molecules, and not the collapse of $\Delta\psi$, could be the true link between mitochondria and apoptosis (Lemasters et al., 1998). On the other hand, significant portions of the Bcl-2 protein are also integrated either into the membranes of the endoplasmic reticulum (ER) or into the nuclear envelope. ER-targeted Bcl-2 retained the ability to block apoptosis in some experimental models (Zhu et al., 1996), suggesting that Bcl-2 could possess some non-mitochondrial, still-unknown mechanisms for promoting cell survival. In this perspective, the finding that some fluorescent probes whose accumulation occurs mostly on ER membranes rapidly lose fluorescence intensity during early phases of apoptosis may suggest that these organelles, and not only mitochondria, are precociously involved in cell-death regulation. Finally, it has been shown that none of the following events represents a point of no return in the apoptotic process: loss of $\Delta\Psi$, changes in mitochondrial permeability transition (MPT), or mitochondrial swelling (Minamikawa et al., 1999), taking into ac-

count that intact human osteosarcoma cells may undergo MPT and mitochondrial swelling in a fully reversible manner, without inducing cell death.

In any case, the analysis of $\Delta\Psi$, a reliable mirror of the capacity to synthesize ATP and of mitochondrial membrane impermeability, is an important parameter to considered when studying apoptosis.

INTERACTIONS BETWEEN HIV, ANTIRETROVIRAL THERAPIES, AND MITOCHONDRIA

More than 10 years ago, Flomembaum et al. studied portions of myocardium obtained postmortem to ascertain whether cardiac tissue from patients who died from acquired immunodeficiency syndrome (AIDS) might be infected by the human immunodeficiency virus (HIV) type-1 (Flomenbaum et al., 1989). The three hearts they analyzed were positive for HIV-1 DNA sequences, and the researchers performed electron microscopy to localize the virus. They described as ''unexpected'' the finding that large numbers of proliferating multilamellated membrane bodies predominantly associated with mitochondria were identified in myocytes contained in these organs, as well as in the heart of an HIV-positive individual. Immunocytochemistry of membrane bodies did not reveal the presence of p24 or gp120 HIV antigens. After this pivotal report, Dalakas et al. showed that either HIV infection or treatment with zidovudine (azidothymidine, AZT), the first antiretroviral drug that consistently increased survival in HIV-infected persons, was capable of causing myopathy (Dalakas et al., 1990). A retrospective study of 20 HIV-positive patients with myopathy (15 treated with AZT and 5 individuals naive for antiretroviral therapy) revealed that such myopathy had inflammatory features and was characterized by the presence of degenerating fibers, cytoplasmic bodies, and endomysial infiltrates consisting of $CD8^+$ cells and macrophages. However, numerous ''ragged-red'' fibers, indicative of abnormal mitochondria with paracrystalline inclusions, were found in the biopsy specimens from the AZT-treated patients but not in those from the others. Together with the observation that suspending therapy resulted in a remarkable histological improvement, it was suggested that long-term treatment with AZT could cause a mitochondrial myopathy.

The presence of ragged-red fibers was then found in association with ubiquitous abnormal mitochondria, that proliferated at the subsarcolemmal level and had marked variations in size and shape accompanied by proliferation and disorganization of their cristae (Pezeshkpour et al., 1991). As AZT is a chain terminator that inhibits mitochondrial γ-DNA polymerase, the amount of mitochondrial (mt) DNA was also measured, and it was observed that AZT-treated patients showed a significant reduction of mtDNA, likely caused by the drug as such rather than by the virus (Arnaudo et al., 1991).

After these reports, several researchers focussed their attention on the role of different antiretroviral drugs, the first of which was AZT, in causing mitochondrial damages. Magnetic resonance spectroscopy suggested impaired

mitochondrial function in AZT-treated HIV-infected patients. In vivo studies using the rat model were indeed very useful to define pathogenetic molecular, biochemical, and ultrastructural toxic events in skeletal muscle, and supported clinical and in vitro findings (Lewis et al., 1992). Electron microscopy was used to analyze patients in whom a 16-week AZT treatment was supposed to provoke either hepatic or muscle toxicity, and such techniques could demonstrate characteristic enlarged mitochondria with paracrystalline inclusions, allowing the distinction of myopathies caused by either HIV or zidovudine (Chen et al., 1992). Flow cytometry was finally used to assess apoptosis and proliferative response to polyclonal mitogens in cells from macaques infected with the simian immunodeficiency virus (SIV), and combined with assessment of the mitochondrial metabolic activity of freshly isolated blood mononuclear cells to determine a novel three-parametric staging system that provided a new prognostic tool in the longitudinal study of SIV infection (Del Llano et al., 1993).

Mitochondria were then studied to ascertain whether HIV had the capacity to damage these organelles directly. Using in situ hybridization detected by electron microscopy and cellular fractionation, researchers found viral RNA in significantly increased amounts in mitochondria relative to the cytoplasm and nucleus (Somasundaran et al., 1994). In contrast, cellular poly(A) RNA or viral Gag proteins were not increased in the mitochondria. Concomitant with HIV expression was a decrease in mitochondrial viability. An inverse relationship between the amount of viral RNA and mitochondrial integrity was demonstrated using immunofluorescent markers to detect probes for HIV RNA transcripts and antibodies to mitochondrial proteins simultaneously in single cells. High levels of viral RNA in mitochondria were present in acutely (but not chronically) infected cells, suggesting that HIV RNA import into mitochondria can compromise mitochondrial function.

The role of proinflammatory cytokines, capable of increasing HIV production (Poli et al., 1990a, 1990b, 1994), was analyzed by immunocytochemistry for interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α on frozen muscle biopsy specimens from HIV-infected patients with various myopathies and from seronegative individuals, some of whom had mitochondrial cytopathies (Gherardi et al., 1994). HIV-infected patients showed positive reactivities in vessels (IL-1) and in inflammatory cells (IL-1 and TNF-α). In AZT myopathy, a majority of fibers showed mild to marked IL-1 expression, which was much weaker in the other mitochondrial myopathies. IL-1β messenger RNA was demonstrated in muscle fibers by in situ hybridization, implying that IL-1 was produced in muscle cells. Immunoelectron microscopy showed that IL-1α was mainly bound to mitochondrial membranes in AZT fibers. Proinflammatory and destructive effects of the studied cytokines might be responsible for several myopathological changes observed in HIV-infected patients, including inflammation and hemosiderin deposits in muscle tissue, and prominent myofibrillar breakdown in AZT fibers.

To examine the long-term effects of AZT exposure on representative precursors of immune cells, investigators cultured human T-lymphocytic H9 cells

or HL-60 promyelocytic cells in the presence of AZT for several months (Agarwal and Olivero, 1997; Nusbaum and Joseph, 1996). Incorporation of AZT into DNA was demonstrated in both cell lines by several methods, including radioimmunoassay, electron microscopy, and immunohistochemistry. In H9 cells, chromosomal aberrations and micronuclei were scored and intracellular lipid distribution was determined: treated cells showed an increase in chromosomal aberrations and nuclear fragmentation compared with unexposed H9 cells. Electron microscopy revealed mitochondrial damage and an elevated accumulation of neutral intracellular lipid deposits, probably as a consequence of a distortion in the beta-oxidation of fatty acids normally carried out by this organelle. These data suggested that patients who took AZT for prolonged periods are significantly disposed to develop cytotoxic phenomena in bone marrow because of both chromosomal and mitochondrial DNA damage. Finally, the ability of transplacental AZT to cause oxidative damage in the nuclear DNA of fetal tissues was tested in a mouse model; significant increases in 8-oxo-2′-deoxyguanosine (8-oxo-dG) were found in the livers, a target tissue for transplacental carcinogenesis, and in the kidneys, but not in the brain or lungs (Bialkowska et al., 2000). Moreover, an increase in ROS could contribute to the mechanism of transplacental carcinogenesis by AZT in mice, and this may also occur in primates.

The concomitant occurrence of muscular and hepatic disturbances and lactic acidosis has been studied in a 57-year-old HIV^+ patient who had been treated with AZT for 3 years and had developed fatigue, weight loss, and lactic acidosis (Chariot et al., 1999). The patient became confused and febrile and died 8 days after detection of high blood lactic acid. Liver biopsy showed diffuse macrovacuolar and microvacuolar steatosis. A muscle sample obtained at autopsy showed mitochondrial abnormalities with ragged-red fibers and lipid droplet accumulation. Southern blot analysis showed depletion of mitochondrial DNA, affecting skeletal muscle and liver tissue but not myocardium and kidney, suggesting that AZT can induce mitochondrial multisystem disease. These observations were confirmed by the fact that independent studies on livers of AIDS patients showed the presence of relevant mitochondrial alterations (Radovanovic et al., 1999).

The role of oxygen-mediated damage in AZT-induced toxicity has been evaluated (de la Asuncion et al., 1998), and it was shown that antioxidants or L-carnitines are capable of protecting cells of different types, such as neurons, myocytes, or lymphocytes (de la Asuncion et al., 1998; Virmani et al., 1995).

Studies on human lymphocytes have demonstrated that cells from patients with HIV infection, cells that are known to be more susceptible to apoptosis, can present mitochondrial damages, either ex vivo or after short-term culture (Carbonari et al., 1997; Castedo et al., 1995; Cossarizza et al., 1997; Macho et al., 1995). Interestingly, recent data indicate that not only the entire virus but also viral proteins are sufficient to induce apoptosis and mitochondrial damages (Jacotot et al., 2000).

DO MITOCHONDRIA PLAY A ROLE IN LIPODYSTROPHY?

Fatty acids derived from hydrolysis of triglicerides circulate in the plasma bound to albumin and are usually removed from blood into mitochondria, to undergo a process called beta-oxidation that ultimately generates ATP. This process is preceded by their transformation into acetyl-CoA via an ATP-dependent reaction that takes place in the microsomal system. Alterations of lipid metabolism provoked by a mitochondrial dysfunction could impair such a biochemical pathway, and thus could have a crucial role in the phenomenon of fat redistribution, or lipodystrophy, an important pathology that has been linked to highly active antiretroviral therapy (HAART). It has been hypothesized that, in some patients, HAART can be responsible for the onset of a characteristic syndrome named lipodystrophy, with peripheral fat wasting and central adiposity (Brinkman et al., 1999). HIV-1 protease inhibitors are generally believed to be the causal agents, although the syndrome has also been observed with protease-inhibitor-sparing regimens. The mitochondrial toxicity of nucleoside-analog reverse-transcriptase inhibiting (NRTI) antiretroviral drugs could play an essential part in the development of this lipodystrophy, similar to the role of mitochondrial defects in the development of multiple symmetrical lipomatosis.

Some years ago, it was shown that a widely used NRTI with a crucial role in anti-HIV therapy, i.e., zidovudine (AZT), was able to act directly on mitochondria because of its capability to integrate into the mitochondrial genome, in a manner similar to what occurs for the nuclear genome. However, if damages to nuclear DNA can be repaired in most cells by an adequate set of enzymes, such activity, which has been acquired during evolution, is likely not present (or, at least, is not particularly efficient) in mitochondrial DNA. As a consequence, damages to mitochondrial DNA, a molecule that is present in several copies in one single organelle and contains genes for crucial enzymes of the respiratory chain, cannot always be repaired and, in turn, can be responsible for alterations in energy metabolism.

Concerning lipodystrophy, scientists have only recently devoted attention to the mechanisms of action of protease inhibitors (PI), the most recent drugs used for HAART, as well as to their interaction with other antiretroviral compounds. Indeed, if on the one hand it is known that some drugs not belonging to the PI category can directly affect mitochondria (as described above), on the other hand it has been shown that the PI are also able to induce apoptosis in adipocytes, a phenomenon that is mediated by the intracytoplasmic block of retinoic-acid binding protein-1, normally responsible for cell differentiation. PI are also capable of interfering with the synthesis of 9-cis retinoic acid, catalyzed by cytochrome P450-3A. Both these mechanisms, which basically concern retinoic acid signaling, clearly cannot explain the onset of lipodystrophy in patients who have never been treated with PI. Thus, other drugs, and in particular NRTI, have been claimed to be directly responsible.

In this regard, one of the most studied compounds was d4T, but few and contrasting data exist on its capacity to affect mitochondrial function and DNA. In hepatoma cells, Pan-Zhou et al. found no effects on the synthesis of 11 polypeptides encoded by mtDNA (Pan Zhou et al., 2000); in PC-12 cells (of neuronal origin), Cui et al. described a dose-dependent inhibition of neurite regeneration but no effects on mtDNA synthesis (Cui et al., 1997). According to Faraj et al., who studied different antiretroviral compounds, d4T is the least potent NRTI capable of inhibiting proliferation of cells belonging either to the granulocyte-monocyte or erythroid lineages (Faraj et al., 1994). In CEM cells (of lymphocytic origin), cell viability and mtDNA content in cultures treated with d4T were significantly reduced in a concentration-dependent fashion compared with cell viability and mtDNA content in untreated cultures (Medina et al., 1994); such cells also showed significant changes in their mitochondrial morphologies, including distortion and reduction of the *cristae* and numerous vesicles.

However, even if direct responsibility for drug-induced mitochondrial damages in the pathogenesis of lipodystrophy has been hypothesized, there is still a substantial lack of direct analysis of the functionality of these organelles, for example, in cells taken from lipodystrophic patients or in other models. Two recent reports described the features of mitochondria in these patients. In the first, Shikuma et al. have performed a cross-sectional evaluation of tissue-specific mtDNA in HIV-infected individuals with lipodystrophy, taking HAART for at least 6 months, in comparison with other cohorts of HIV^+ patients (Shikuma et al., 2001). They performed semiquantitative analysis of mtDNA content in subcutaneous fat biopsies taken from the neck, abdomen, and thigh. They found that decreased mtDNA levels were present in subcutaneous adipose tissue from lipodystrophic patients on NRTI-containing HAART; however, no large mtDNA deletions or insertions were found in any specimen. Viganò et al. showed that, as far as changes in $\Delta\psi$ or tendency to undergo apoptosis were concerned, peripheral lymphocytes from lipodystrophic children did not display significant differences from those of healthy control or HIV-infected children without lipodystrophy (Viganò et al., 2001). The latter study is based upon some cytometric technologies described below, which thus could be useful, at least in part, for clarifying the role of mitochondrial damages during HIV infection and its therapy.

MITOCHONDRIAL FUNCTIONAL PARAMETERS THAT CAN BE DIRECTLY ANALYZED IN LIVING CELLS BY CYTOMETRIC METHODS

Mechanisms that regulate changes in membrane potential of organelles with a negative interior, as depicted in Figure 13.1, have been analyzed with several membrane-permeable, lipophilic cations, because of their property to accumulate in such organelles and/or liposomes. Such probes include those

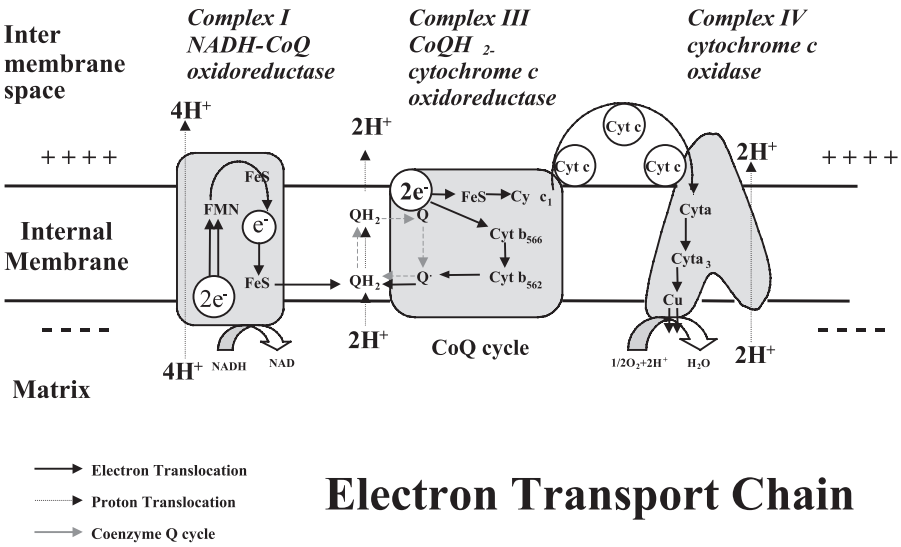

Figure 13.1. Schematic representation of events that occur in the mitochondrial inner membrane during the production of membrane potential.

that exhibit optical and fluorescence activity after accumulation in energized systems [e.g., 3,3′-dihexyloxadicarbocyanine iodide, nonyl acridine orange (NAO), safranine O, rhodamine-123 (Rh123), etc.], radiolabelled probes, (e.g., [^{3}H]methyltriphenyl-phosphonium, etc.), and unlabelled probes used with specific electrodes [e.g., tetraphenylphosphonium ion (TPP^+), etc.]. These systems have the following possible disadvantages:

1. time required to achieve equilibrium distribution of a mitochondrial membrane probe;
2. degree of passive (nonspecific) binding of probes to a membrane component, as in the case of NAO, which detects mitochondrial mass as it binds to cardiolipin (Maftah et al., 1990), or Rh123, which has several energy-independent binding sites (Lopez-Mediavilla et al., 1989);
3. toxic effects of probes on mitochondrial functional integrity;
4. sampling procedures;
5. interference from light-scattering changes and from absorption changes of mitochondrial components; and
6. requirement for large amounts of biological materials.

The TPP electrode affords an easy and precise tool to measure $\Delta\psi$ owing to the low interference between bound TPP^+ and the membrane, and to the electrode lack of response to species other than TPP^+. However, this method requires "discrete" amounts of biological samples (on the order of several million

cells for each experimental point) and, in contrast to isolated mitochondria, the uptake of this lipophilic cation by several intact mammalian cells is a slow process.

A few years ago, using the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), we developed an original cytofluorimetric technique to detect variations in $\Delta\psi$ at the single-cell level (Cossarizza et al., 1993), which has been validated at the single-mitochondrion level (Cossarizza et al., 1996). This probe allows us to analyze $\Delta\psi$ not only flow cytometry but also by confocal microscopy (Bednar et al., 1999; Ceruti et al., 1997; De Maria et al., 1997; Dispersyn et al., 1999; Franceschi et al., 1996; Guidarelli et al., 1995; Gorman et al., 1997; Salvioli et al., 2000b; Shenker et al., 1999; Trimmer et al., 2000). JC-1 has an advantage over rhodamines and other carbocyanines capable of entering selectively into mitochondria, because it reversibly changes color from green to orange as membrane potential increases, i.e., as the mitochondrial membrane becomes more polarized, over values of about 80–100 mV (Reers et al., 1991; Smiley et al., 1991). This property, demonstrated in Figure 13.2, is due to the reversible formation of JC-1 aggregates, which causes a shift in emitted light from 530 nm (emission of JC-1 monomeric form) to 590 nm (emission of J-aggregate), when excited at 490 nm. Both colors

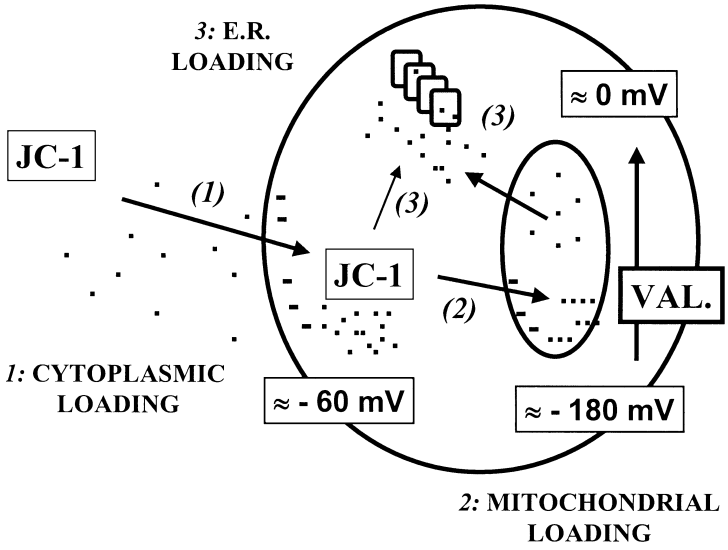

Figure 13.2. Intracellular distribution of JC-1 before and after the depolarization of mitochondria with valinomycin (*VAL.*). Note that during the staining with this dye, three main events occur: 1) cytoplasmic loading, which is dependent upon plasma membrane potential—the probe remains in monomeric form, i.e., green; 2) mitochondrial loading, dependent upon $\Delta\psi$—JC-1 forms aggregates and turns orange; and 3) endoplasmic reticulum (ER) loading, that theoretically can take place after the fall in $\Delta\psi$. In the case of JC-1, aggregates are no longer present when the dye is released from mitochondria, and the dye becomes green again.

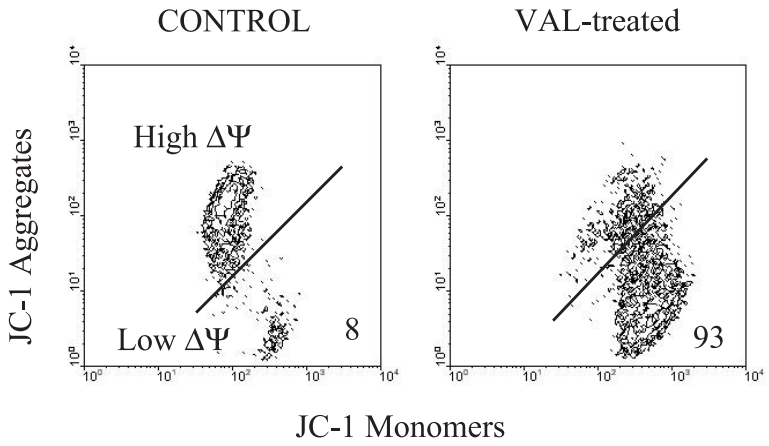

Figure 13.3. Depolarization of mitochondria in U1 cells after treatment with valinomycin (*VAL*). Numbers indicate the percentage of cells with depolarized mitochondria, present in the lower right part of the panels.

can be detected using the filters commonly mounted in flow cytometers or confocal microscopes, so that green emission can be analyzed in fluorescence channel 1 (FL1) and greenish orange emission in channel 2 (FL2). JC-1 is mostly qualitative, considering the shift from green to orange fluorescence emission and vice versa, but can also give quantitative information on mitochondrial mass—even if less precise than those provided by probes such as MitoTracker Green, or nonyl acridine orange at low doses—considering the pure fluorescence intensity, which can be detected in both FL1 and FL2 channels. Figure 13.3 shows the basic fluorescent changes of the probe after loading of the HIV-1-infected cell line U1 (derived from the myelomonocytic clone U937, cultured in the presence of the virus (Folks et al., 1987, 1988)) with JC-1, and its treatment with the depolarizing agent valinomycin. The behavior of such cells is absolutely similar to that of the parental cell line U937 (Salvioli et al., 2000a), indicating that, in this HIV-infected line, mitochondria respond in the same manner to depolarizing agents.

STUDIES ON MITOCHONDRIA IN HIV-1 INFECTED CELLS

In the past few years, we have studied the behavior of mitochondria during apoptosis in a variety of experimental models (Cossarizza, 2000; Cossarizza et al., 1994, 1995a, 1995b, 1999; Troiano et al., 1998; Tropea et al., 1995), as well as during acute HIV infection. In the first study, we analyzed the presence of $\Delta\psi$ alterations and the propensity to undergo apoptosis in peripheral blood lymphocytes from subjects with acute HIV syndrome, and evaluated whether antioxidant drugs that can be used in therapy, such as N-acetyl-cysteine (NAC), nicotinamide (NAM), or L-acetyl-carnitine (LAC) were able to reduce the

damages (Cossarizza et al., 1997). Significant $\Delta\psi$ alterations and a high tendency to undergo spontaneous apoptosis were present in PBL from the subjects studied, but the presence of NAC, NAM, or LAC was able to rescue most cells from apoptosis. The changes in $\Delta\psi$ and apoptosis observed in lymphocytes collected in the earliest phases of the acute viral syndrome decreased significantly after a few days, even in the absence of antiretroviral therapy. Significant correlation was found between spontaneous apoptosis and TNF-α or p24 plasma levels, as well as between apoptosis and the percentages of circulating $CD4^+$ or $CD8^+$ T cells. It was suggested that NAC, NAM, or LAC could rescue cells through a protective effect on mitochondria, a well-known target for the action of TNF-α and ROS, the production of which is strongly induced by this cytokine.

In a second paper, we described the role of the CD95/CD95L system during primary infection, analyzing the functional characteristics of lymphocytes in 12 patients during the acute phase of viral infection and in the following months (Cossarizza et al., 2000). Almost all cells, including $CD8^+$ T cells with a virgin phenotype, B lymphocytes, and natural killer (NK) cells, displayed CD95 molecules on the plasma membrane. Activation of CD95 on the surface of isolated lymphocytes by anti-CD95 monoclonal antibodies or binding to CD95L induced rapid apoptosis that was accompanied by relevant changes in $\Delta\psi$. We showed that during acute, primary infection, a prolonged deregulation of the CD95/CD95L system may exist, which provokes mitochondrial damages and relevant apoptotic phenomena. Such deregulation is likely not entirely related to virus production, but may contribute to the pathogenesis of the disease. It was pointed out that mitochondria are involved in the complex balance existing between proapoptotic events (increase in CD95 expression), likely triggered by the host as a method to limit viral production, and antiapoptotic events (decrease in CD95L expression), likely triggered by the virus as a way to increase its production and survival.

Concerning chronic HIV infection, we have focused our attention on a cellular model of in vitro infection. In this chapter, we show some cytofluorimetric studies on mitochondrial membrane potential and apoptosis in a cell line chronically infected with HIV-1, U1, used for many years as a model for studies on the physiopathology of infected cells (Griffin et al., 1989; Kalebic et al., 1991; Poli et al., 1994; Pomerantz et al., 1990). We have analyzed the capacity of such cells to undergo apoptosis after treatment with TNF-α and cyclohexamide (CHX), two classical agents used to induce cell death in the U937 cell line (Cossarizza et al., 1995b).

DYES, CHEMICALS, AND CELL CULTURES

JC-1 and MitoTracker™ Red CMXRos (MT) were from Molecular Probes (Eugene, OR). JC-1 was dissolved in N,N-dimethylformamide (DMF) at a concentration of 1 mg/mL and stored at −20 °C ; MT was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 μM and stored at −20 °C.

TNF-α, CHX, and propidium iodide (PI) were from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated annexin-V (ANX-V) was from Bender MedSystem (Vienna, Austria).

Micoplasma-free U937 or its HIV-infected clone U1 was cultured in complete medium (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) and kept at 37 °C in a humidified atmosphere (5% CO_2 in air). Cells were collected during the log phase of growth, washed in phosphate buffered saline (PBS) at room temperature, counted, and adjusted to a concentration of 10^6/ml. Cells were then treated with 4 mM CHX for 2 h, and subsequently with 50 IU/ml TNF-α for 4 or 6 h. At the end of the incubation period, cells were harvested, washed, counted, and stained as described below.

FLOW CYTOMETRY

The analysis of the samples was performed using a FACScan cytometer (Becton Dickinson, San Jose, CA) equipped with an argon-ion laser tuned at 488 nm. The following parameters were assessed.

Mitochondrial Membrane Potential ($\Delta\psi$)

Cells were stained with the lipophilic cationic probe JC-1 (Molecular Probes) as described (Cossarizza et al., 1993). Staining with this fluorescent probe is, in principle, quite simple. For U1 cells, after incubation with apoptogenic agents, a cell suspension is adjusted to $0.5–1 \times 10^6$ cells/ml and incubated with 2.5 μg/ml JC-1 in complete medium (RPMI-1640 with 10% FCS) for 10 min at room temperature in the dark. During the addition of the dye, or immediately after, it is important to vortex the tubes, as the solubility of the probe is low in water; in this manner aggregates of the probe should not be present or should disappear. Then, the cells are washed twice in PBS, resuspended in PBS (it is also possible to use PBS with 0.5% paraformaldehyde), and analyzed. It is advisable to prepare a functional ''negative''control, treating a parallel sample with drugs able to collapse $\Delta\Psi$, such as the K^+ ionophore valinomycin (100 nM or more) or the proton translocator carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, 250 nM). A dramatic change of the fluorescence distribution occurs (as in Fig. 13.3), which indicates where cells are whose mitochondria have a low $\Delta\psi$. The compensation of FL1 and FL2 either in control or treated samples is then set.

Changes in $\Delta\psi$ and Early Apoptosis by Dual Staining with MT and ANX-V

Thirty minutes before the end of the scheduled incubation period, 200 μl cell suspension is harvested from each Petri dish, transferred to a suitable test tube, and the volume is brought to 1 ml with complete medium. These samples are

stained with 50 nM MT for 30 min at 37 °C. At the end of the incubation time, the samples are washed once in PBS and resuspended in 200 μl ANX-V binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), stained with 5 μL ANX-V, and incubated for 10 more minutes at room temperature (Salvioli et al., 2000b). The cells are then washed with binding buffer to remove the excess ANX-V, resuspended in PBS (eventually containing paraformaldehyde), and analyzed. MT and ANX fluorescence are collected in FL2 and FL1 channels, respectively.

Late Apoptosis

The classical appearance of the hypodiploid peak of PI fluorescence, according to Nicoletti et al. (Nicoletti et al., 1991), is utilized for the quantification of this parameter, as described (Barbieri et al., 1992). Briefly, cells are resuspended in hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml PI, and kept for 20 min at 4 °C. Then, cells are analyzed on the flow cytometer, and those with low PI fluorescence, i.e., that contain less DNA, and with the typical change in physical parameters (as revealed by an increase in the side scatter) are considered apoptotic.

A minimum of 10,000 cells per sample are acquired in list mode and analyzed with WinMDI 2.8 software (kindly provided by Dr. J. Trotter, Scripps Institute, La Jolla, CA).

INTERPRETATION OF THE DATA

Many mechanisms can be triggered that provoke the death of immune cells, including a partial, incomplete activation (i.e., delivery of the first stimulatory signal, which is not followed by the second), the action of pro-inflammatory cytokines such as TNF-α, and the activation of receptors such as CD95 that mediate apoptosis, among others (Buttke and Sandstrom, 1994; Collins et al., 1991; Dive et al., 1992; Falcieri et al., 1993; Green et al., 1992; Grell et al., 1989; Nagata, 1999; Nagata and Golstein, 1995). However, one of the key problems that await a solution concerns the individual sensitivity of infected cells to apoptosis. As a consequence, much less is clear concerning apoptosis in infected lymphocytes or monocytes, and studies with chronically infected cell lines are critical to understand the interactions between virus and host that cause or inhibit cell death. On the one hand, apoptosis is a defense system that cells activate to destroy agents that are dangerous for the organism. On the other hand, undesired invaders like viruses gain no advantage in provoking death of the infected cell, and have thus developed antiapoptotic mechanisms. Recently, using chronically infected cell lines such as U1 and ACH-2 (derived from the lymphocytic clone A301), we studied the expression of CD95 (APO-1/Fas), a member of the TNF receptor family that is deeply involved in this process, and its ligand (CD95L) (Pinti et al., 1999). Flow cytometry revealed that

the lymphocytic cell line A301 and its infected clone ACH-2 mounted the same amount of plasma membrane CD95. In contrast, HIV-infected U1 cells had a consistent decrease in membrane CD95 expression compared with the parental monocytic line U937. Using an original approach based upon competitive-quantitative (QC) polymerase chair reaction, we then studied the mRNA levels of CD95 ligand, CD95L, and we could observe that ACH-2 expressed much less CD95L mRNA than the parental cell line A301, while in both U937 and U1 CD95L mRNA levels were below the limit of detection, which was on the order of 2000 copies/RNA per million cells (Pinti et al., 1999). To further investigate the functionality of CD95/CD95L system, we quantified the expression of different mRNAs for the total or the membrane form of CD95, the modulation of such mRNAs, the protein expression on the cell membrane, and the capacity of infected cell lines of different origin to trigger apoptosis after activation of CD95 pathways, as well as under a variety of conditions (Pinti et al., 2000). We found that infected cells of monocytic origin preferentially produce the "protective" (soluble) form of CD95, and not any detectable CD95L mRNA, whereas lymphoid cells produce more mRNA for the membrane form of CD95 (which triggers apoptosis) along with reduced but detectable amounts of CD95L mRNA. It is possible to hypothesize that, as far as the CD95/CD95L system is concerned, a complex balance exists between proapoptotic events, probably triggered by the host to limit viral production, and antiapoptotic events, probably triggered by the virus to increase its production and survival. In cells of monocytic origin, which act as reservoir for the virus, the antiapoptotic molecules are favored; in cells of lymphocytic origin, molecules with an apoptotic meaning are prevalent.

Further, we performed functional studies and analyzed the capability of one of these cell lines, U1, to display mitochondrial damages and undergo apoptosis after stimulation through a classical pathway utilized in human cells of monocytic origin, that of TNF-α. Here we show that, as illustrated in Figure 13.4, U1 cells have a marked decrease in sensitivity to that apoptotic pathway. Indeed, staining with JC-1 showed that after 6 h of treatment with TNF-α, the percentage of cells with relevant mitochondrial depolarization was much lower than that of the parental cell line, U937. This was confirmed in parallel samples by the analysis of samples stained with MitoTracker Red and annexin V (Fig. 13.5), or with PI (Fig. 13.6). In this case, too, U1 cells showed less mitochondrial damage or apoptosis than the parental line U937 (not shown).

FUTURE DIRECTIONS: CYTOFLUORIMETRIC ANALYSIS OF INTRAMITOCHONDRIAL CARDIOLIPIN DISTRIBUTION

Recent studies have suggested that cardiolipin (CL), a diacidic phospholipid with an unusually high content of linoleic acid ester residues that is isolated from beef heart (Hatch, 1996), could be involved in apoptotic cell death (Lieser et al., 1998; Ushmorov et al., 1999). CL is present throughout eukaryotes, in-

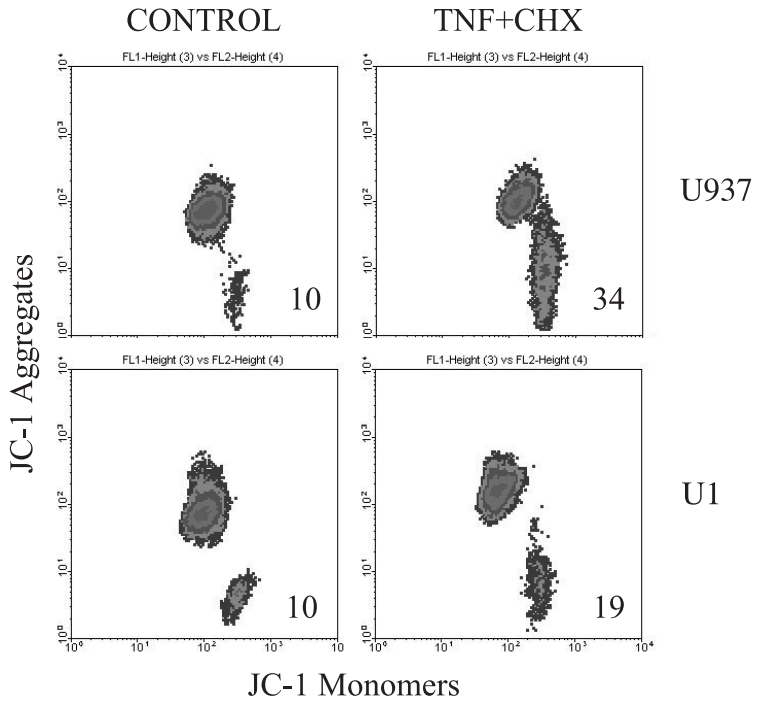

Figure 13.4. Effects of TNF-α and CHX on mitochondria in U937 and U1 cells. Note that HIV-infected cells display a lower sensitivity to the apoptotic agents used. Numbers indicate the percentage of cells with depolarized mitochondria, present in the lower right part of the panels.

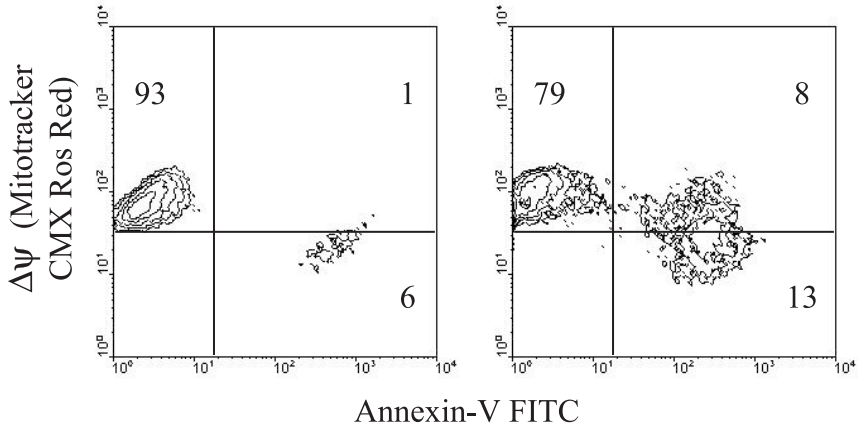

Figure 13.5. Analysis of early apoptosis (using Annexin-V FITC) and mitochondrial membrane potential (analyzed with MT) in U1 cells after 2 h of incubation with CHX followed by 4 h with TNF-α. Numbers indicate the percentage of cells with high Δψ and negative Annexin-V (*upper left quadrant*), with high Δψ and positive to annexin V (*upper right quadrant*), or with low Δψ and positive for annexin V (*lower right quadrant*). In a parallel experiment, the percentage of U937 in the upper right quadrant was 14%, that in the lower right quadrant was 22%.

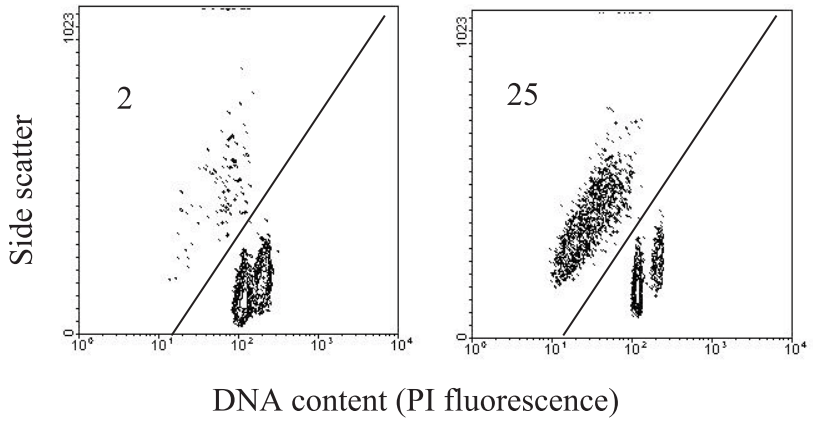

Figure 13.6. Analysis of apoptosis in U1 cells. Numbers indicate the percentage of apoptotic nuclei, present in the higher left part of the panels; in a parallel experiment, apoptosis in U937 cells was 40%.

cluding animals, plants, and fungi. In mammalian tissues and in yeast, CL is found exclusively in mitochondria (Ioannou and Golding, 1979; Schlame and Hostetler, 1997), a site in which it is specific and essential for the normal function of the mitochondrial inner membrane system, and, in particular, for i) the activity of cytochrome bc1 and cytochrome c oxidase, key components of the electron transport chain; ii) the attachment of cytochrome c to the inner mitochondrial membrane; iii) the activity of mitochondrial transporters, including adenosine diphosphate/ATP translocase, the mono-, di-, and tri-carboxylate carriers, the α-ketoglutarate, aspartate/glutamate, and palmitoylcarnitine carriers, and the (acyl)carnitine translocase system; iv) the functionality of F_0F_1-ATP synthase (Gomez and Robinson, 1999).

We have recently developed a cytofluorimetric approach to study intramitochondrial cardiolipin transverse distribution in intact cells (Garcia Fernandez et al., 2000). The assay is based upon the following peculiar properties of the fluorescent dye 10-N-nonyl-3,6-bis(dimethylamino)acridine (Nonyl Acridine Orange, NAO): i) two molecules of NAO can bind with high affinity one single CL molecule, forming NAO dimers; ii) the dye is unable to bind zwitterionic phospholipids and has low affinity for other anionic phospholipids; and iii) NAO is capable of changing its emission because of its spectral properties, as after dimer formation the fluorescence can shift from green (monomeric form) to red (dimeric form) (Petit et al., 1992, 1994). Using such an assay, we first showed that the distribution of CL was markedly different in HCW-2 cells compared with the parental HL-60 line (Garcia Fernandez et al., 2000). We have started the analysis of CL distribution in U1 cells. Preliminary data seem to indicate that no major differences exist between the two cell types, suggesting that infection with HIV-1 is not capable of provoking changes in mitochondrial structure that could be responsible for functional damages.

CONCLUSION

During HIV infection, mitochondria are involved in a variety of pathways and processes. Not only are they crucial for producing the energy required by the cell to survive and maintain homeostasis, but also, acting at different levels, these organelles are capable of inducing or modulating cell death. The techniques we have described in this chapter provide a very useful tool for those investigators interested in studying mitochondria during HIV infection, as they allow functional analysis of intact, living cells.

Moreover, there is a continuous development of new fluorescent probes for the detection of biological parameters related to mitochondrial activity, and it is reasonable to predict that other new methods and techniques will be set up soon. The use of multiple lasers and of even more sophisticated activated cell sorters or confocal microscopes will dramatically increase the number of possible investigations, and in the very near future it will be possible to detect more and more parameters at the single-cell level. With flow cytometry and microscopy systems of high efficiency and new specific probes, it will be of extreme interest to combine the advantages of the cytometric approach with those of molecular biology and to go deeper into the details of the biological processes and mechanisms that regulate cell death and, in general, changes in cell functionality during HIV infection.

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14

Multiple Roles of Cytokines in HIV Infection, Replication, and Therapy

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THE CYTOKINE NETWORK

Cytokines are soluble molecules produced by both immune and nonimmune cells such as endothelial and epithelial cells. When the first biological activities, later defined as ''lymphokines'' and ''monokines,'' were discovered, the general assumption was that each molecule was endowed with a unique biological function. In contrast, it is now well recognized that cytokines are characterized by pleiotropy (one molecule, multiple targets) and redundancy (different cytokines exert similar biological effect on the same cell) (Paul, 1989; Paul and Seder, 1994). In addition, cytokines have usually more than one receptor (chemotactic cytokines, or chemokines, in this regard, are extremely promiscuous), whereas individual cells express multiple receptors for different cytokines, thus allowing complex synergistic or antagonistic interactions.

Cytokines control both innate and specific immune responses, the entire process of inflammation, and its turning-off; they can also exert mitogenic activities or induce growth arrest, cell differentiation, and death by regulating complex apoptotic pathways (Dinarello, 1998). Unlike hormones, they are usually characterized by short-term and self-limited action (autacoids) (Paul, 1989). In this regard, cytokines mediate their effects via high-affinity binding to cell surface receptors, and they can influence cells surrounding the site of production in a paracrine and autocrine fashion.

Cytokine synthesis is tightly controlled at the transcriptional level, but also post-transcriptionally. In fact, many cytokine mRNAs contain adenosine uridine (AU)-rich regions whose presence or removal strongly influences their half-lives and, ultimately, the levels of cytokine production (Revel and Groner, 1978). Conversely, cytokines profoundly affect several cellular genes by influencing both transcriptional and post-transcriptional events (Arai et al., 1990).

The biochemical pathways transducing cytokine signals to the nucleus have been elucidated only recently. Many cytokines utilize the Janus kinases and signal transducer and activator of transcription (JAK/STAT) pathway. Activation and dimerization of different STATs is responsible for specific cytokine effects (Ihle, 1995; Schindler and Darnell, 1995).

Cytokines are classified as being either pro- or anti-inflammatory, or immunoregulatory if they exert differential effects on Th1 vs. Th2 cell polarization (O'Garra and Murphy, 1996). Typical examples of pro-inflammatory cytokines are tumor necrosis factor-α (TNF-α) or TNF-β (renamed lymphotoxin-α) and interleukin-1α/β (IL-1α/β), whereas transforming growth factor-β (TGF-β), IL-4, and IL-10 are potent anti-inflammatory molecules. In addition, IL-4 induces the differentiation of and is secreted selectively by Th2 cells, devoted to the humoral immune response. On the other hand, IL-12 and interferon-γ (IFN-γ) are the principal inducer and product of Th1 cells, respectively, leading to phagocyte-dependent cell-mediated immune response. Both IL-12 and IFN-γ also exert pro-inflammatory effects (O'Garra and Murphy, 1996; O'Garra et al., 1997).

Cytokines are very important components of our immune defense against pathogens and cancer cells; however, their dysregulated production can also be involved in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis or autoimmune thyroiditis. In addition, genetic diseases leading to primary immunodeficiencies frequently involve either cytokines or their receptors (Jouanguy et al., 1999). On the other hand, cytokines or pharmacological agents aimed at the suppression of their expression (such as cyclosporin A or FK506) are experimentally used in different diseases, including human immunodeficiency virus (HIV) disease, as discussed later, although still with great difficulties caused by prominent side effects (Chapuis et al., 2000).

Cytokines can be measured in all body fluids as well as in whole blood. Enzyme-linked immunosorbent assay (ELISA) is the most common method of detection of cytokines present in plasma, urine, and other secretions, or in culture supernatants. Intracellular staining of cells maximally stimulated and treated with inhibitors of secretion such as brefeldin-A allows the analysis of cytokine expression by cytofluorimetry. By this approach, patterns of cytokine expression can be interpolated with those of other cell-associated markers. Cytokine detection has become an important tool for investigating major histocompatibility complex (MHC)-restricted cytolytic T lymphocytes (CTL) activities by the ELISPOT methodology. Finally, multiple molecular approaches, from conventional to real-time polymerase chain reaction amplification to RNase protection assays, can be easily applied to study the expression patterns of sev-

TABLE 14.1. Cytokine Receptor Families and Their Ligands

Cytokine Receptors	Cytokines
Ig superfamily	IL-1, IL-6, PDGF, FGF, M-CSF
Type I (WSXWS)	IL-6, IL-2 (β and γ chains), IL-3, IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF
Type II	IFNs (α, β, γ)
Type III	TNF-α (p55, p75)
7 TMD-R	chemokines
Multi protein complex	IL-2 (α, β, γ), IL-6 (IL-6R + gp130), IL-3 (IL-3R + gp150)

Underlined cytokines utilize different classes of receptors.

eral cytokine mRNAs with limited amounts of biological samples, and therefore these methods are of particular interest in clinical settings.

CYTOKINE RECEPTORS

Cytokine receptors are commonly subdivided into five families based on their secondary structural domains (Table 14.1). The first family contains different extracellular domains (1–3) belonging to the immunoglobulin (Ig) superfamily. In addition to classic cytokines, like IL-1 and IL-6, both macrophage colony-stimulating factor (M-CSF) and growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) utilize these receptors.

The second cytokine receptor group, one of the largest families, and also termed "type I" cytokine receptors, is characterized by an extracellular sequence of five amino acids (tryptophan-serine-X-tryptophan-serin or WSXWS) in close proximity to the transmembrane region of the receptor. An Ig and a WSXWS domain also characterize the IL-6 receptor.

The third group of molecules, "type II" cytokine receptors, is characterized only by its primary sequence. Type II receptors are typically used by IFNs.

TNF/Nerve growth factor (NGF) receptors are also called "type III" cytokine receptors and encompass several regulatory molecules such as CD27, CD30, and CD40. Some members, FAS (CD95) and the p55 TNF receptor, possess a "death intracellular domain" delivering apoptotic signals to the cells.

The fifth family of cytokine receptors consists of seven transmembrane domain receptors, the largest receptor family identified in nature, comprising the chemokine receptors.

Finally, many cytokine receptors are complexes of different transmembrane proteins. For example, the IL-2 receptor has three subunits named α, β, and γ. β and γ subunits contain the WSXWS domain and are responsible for binding and signal transduction; their association with the α subunit (CD25) increases the binding affinity for IL-2 (Minami et al., 1993). The IL-6 receptor interacts with a transmembrane protein, characterized by an Ig and WSXWS domain.

TABLE 14.2. Cytokine Regulation of the HIV Life Cycle

Induction	Suppression	Bifunctional Cytokines
IL-1, IL-3, IL-6, IL-7, IL-12, IL-18, TNFs, CD30L, M-CSF, GM-CSF	IFN-α/β, IL-13, IL-16, MDC*	IL-2, IL-4, IL-10, IFN-γ, TGF-β, MCP-1,† RANTES, MIP-1α, MIP-1β‡

* Monocyte-derived chemokine (MDC) has been described to suppress virus replication in activated PBMC (Pal et al., 1997) and, recently, in MDM (Cota et al., 2000b).

† Monocyte chemotactic protein 1 (MCP-1) has been associated with both inhibitory and enhancing effects in PBMC (Frade et al., 1997; Kinter et al., 1998; Vicenzi et al., 2000).

‡ Although the CC chemokines RANTES, MIP1-α, and MIP1-β are potent suppressors of HIV entry and replication in T lymphocytes (Cocchi et al., 1995), they have also shown inductive effects in both T cells and macrophages (Dolei et al., 1998; Kinter et al., 1998; Schmidtmayerova et al., 1996).

Similarly, IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors are type I receptors that associates with a 150-kD transmembrane subunit endowed with WSXWS domains and transducing the cytokine-mediated signal to the target cells (Taga and Kishimoto, 1992).

CYTOKINE SIGNALING AND INTERFERENCE WITH HIV REPLICATION

Cytokines can enhance or inhibit virus expression from cells infected chronically with HIV. Enhancement of virus replication or expression is the dominant effect of several pro-inflammatory cytokines, such as TNFs and IL-1. In addition, several other cytokines have been described as inducers of virus replication (Al-Harthi et al., 1998; Folks et al., 1987; Gendelman et al., 1988; Koyanagi et al., 1988; Poli et al., 1990; Smithgall et al., 1996) (Table 14.2). On the other hand, only a restricted number of cytokines, namely IFNs (Shirazi and Pitha, 1992, 1993; Shirazi et al., 1994), IL-13 (Montaner et al., 1993, 1997), and IL-16 (Amiel et al., 1999; Baier and Kurth, 1997; Maciaszek et al., 1997; Truong et al., 1999; Zhou et al., 1999) have shown clear-cut inhibitory effects on virus production. Finally, several cytokines can exert either enhancing or inhibitory effects on virus multiplication, depending from the cell type (T lymphocytes vs. macrophages, or primary cells vs. cell lines) or the experimental conditions (e.g., as a function of whether the cytokine was added before, at the same time, or after infection) (Koyanagi et al., 1988; Lazdins et al., 1991; Poli et al., 1991, 1992) (Table 14.2).

Several biological effects caused by cell stimulation with TNF-α are mediated by the activation of transcription factors, such as nuclear factor (NF)-κB and AP1, which also play an important role in HIV expression. NF-κB (a heterodimer typically composed of p50/p65 subunits) is normally present in the

cytoplasm of several cell types in physical association with the inhibitory molecule known as I-κB, which prevents its migration into the cell nucleus (Siebenlist et al., 1994). Several pathways, including activation of protein kinase C, lead to phosphorylation of I-κB and to the consequent dissociation of phosphorylated I-κB from NF-κB (Franzoso et al., 1994). Two binding sites for NF-κB in the core enhancer region of the HIV-1 long terminal repeat (LTR) allow binding of the transcription factor with consequent triggering or potentiation of viral transcription (Fig. 14.1). Of note is the fact that different clades of HIV-1 are characterized by different numbers of NF-κB binding sites in the LTR, whereas HIV-2, and its cognate simian immunodeficiency virus (SIV), possess only 1 kB binding region. Clade C HIV-1, rapidly spreading in several regions of sub-

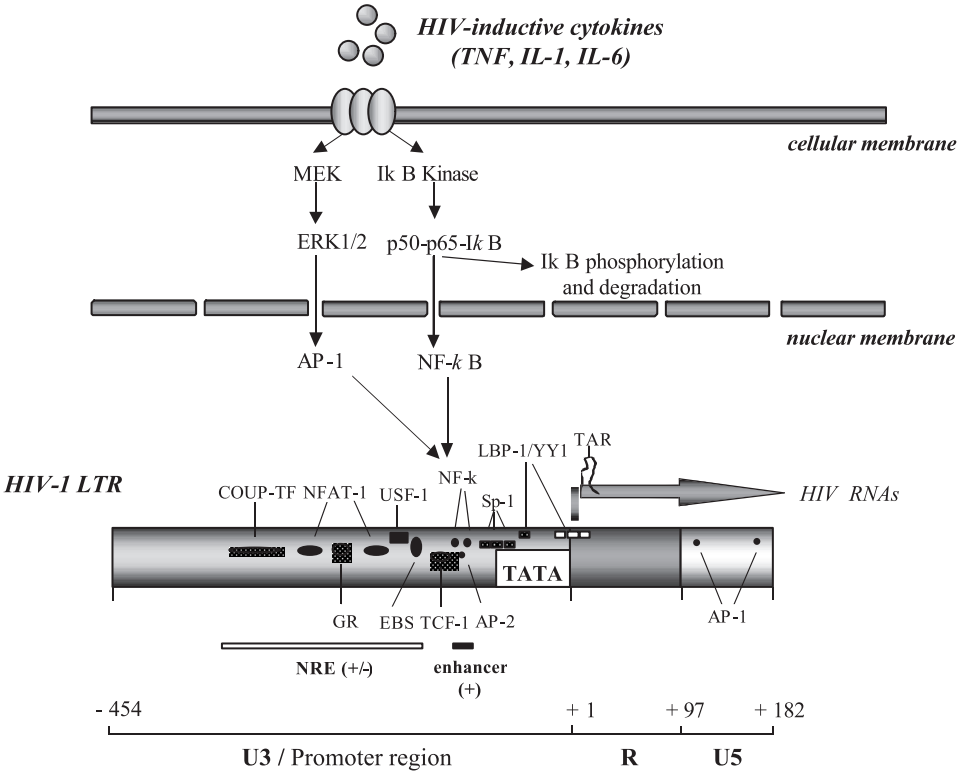

Figure 14.1. Transcriptional control elements of the HIV-1 LTR. The structure of HIV-1 LTR and the binding sites for cellular transcription factors are shown. Enhancer sequences and a region of the HIV-1 LTR known as the negative regulatory element (*NRE*) as well as the Tat responsive element (*TAR*) in the RNA transcripts are shown. *COUP-TF*, chicken ovalbumin upstream promoter transciption factor; *NFAT-1*, nuclear factor of activated T cells; *GR*, glucocorticoid receptor; *USF-1*, upstream stimulating factor; *EBS*, Ets binding factor; T-cell factor (*TCF-1*), (or lymphoid enhancer-binding factor, LEF), *NF-κB*, nuclear factor-κB; AP-1/2, activating protein-1 or 2, composed of heterodimers of Jun and Fos; Sp-1, promoter-specific transcription factor; LBP-1, leader binding protein-1; *YY1*, ying-yang-1.

Saharan Africa, has three binding sites for NF-κB and its replication is more potently upregulated by TNF-α in vitro in comparison to that of other HIV clades with one or two binding sites (De Baar et al., 2000; Jeeninga et al., 2000; Montano et al., 2000).

The presence of functional binding sites for NF-κB in the virus LTR has been usually associated with the total loss of responsiveness to TNF-α, whereas other stimuli, such as phorbol esters, could maintain their effects, at least in part (Antoni et al., 1994). However, more recently, AP1-mediated transcription consequent to the activation of ERK-1, ERK-2 and, MAP kinases has also been demonstrated to activate virus expression in chronically infected U1 cells stimulated with TNF-α (Yang et al., 1999).

Like TNF-α, IL-1 α/β has also demonstrated the capacity of inducing viral replication. However, IL-1 failed to activate NF-κB (Poli et al., 1994b), but likely operates via p38 MAPK in chronically infected U1 cells (Shapiro et al., 1998a). Upregulation of HIV through the activation of p38 MAPK and NF-κB was also observed after stimulation with IL-18, although these studies did not rule out a possible role of endogenous TNF-α and IL-6, cytokines that are also induced by this recently identified cytokine (Shapiro et al., 1998b).

Unlike TNF-α or IL-1β, a post-transcriptional mechanism of up-regulated HIV expression (likely involving modulation of the Rev/RRE axis of HIV RNA nucleus to cytoplasm export) was early identified in U1 cells stimulated with IL-6 (Poli et al., 1990).

In addition to tumor cell lines, it has been formally proven that stimulation by individual cytokines such as IL-2, IL-7, IL-12, or a cytokine combination such as TNF-α plus IL-2 and IL-6 could also induce HIV replication in primary cells infected in vitro and from PBMC and latently infected resting memory $CD4^+$ T lymphocytes infected in vivo with HIV (Chun et al., 1998; Kinter et al., 1995a, b). In this regard, B lymphocytes from infected individuals, known to be polyclonally activated in vivo, are important chaperones of viral particles (Moir et al., 2000) and a source of HIV-inductive cytokines such as TNF-α, IL-6, and, potentially, IL-7 (Benjamin et al., 1994). Indeed, B lymphocytes have been shown to induce viral replication from both chronically infected cell lines and autologous $CD4^+$ T cells of HIV-infected individuals (Rieckmann et al., 1991a, b).

HIV INHIBITORY CYTOKINES

Among the few clear-cut HIV suppressive cytokines, type I IFNs (IFN-α/β and IFN-γ) play an important role. IFN-α, in particular, has shown potent anti-HIV activities in vitro by inhibiting multiple steps of the virus life cycle, including reverse transcription and transcription of integrated provirus in acutely infected primary cells (Poli et al., 1994a). In addition, type I IFNs inhibited the release of new progeny virions from the cell surface in chronically infected cells (''post-budding'' effect) (Poli et al., 1989). Despite a demonstrated antiviral

activity in vivo as a monotherapy agent, and the additional beneficial effects observed in AIDS patients with Kaposi' sarcoma (Lane et al., 1988), IFN-α/β are not usually considered in highly aggressive antiretroviral therapy (HAART) trials, mainly because of their substantial toxicity. However, the availability of PEGylated forms of IFN-α, characterized by a prolonged half-life and easier compliance, may lead to a rediscovery of this classical antiviral molecule.

Similar to IL-4, but produced by macrophages, IL-13 was shown to be a potent inhibitor of HIV replication in monocyte-derived macrophages (MDM) but not in activated T lymphocytes (Montaner et al., 1993, 1997). Furthermore, IL-13, like IL-4, inhibited bacterial lipopolysaccharide-induced HIV expression in GM-CSF-stimulated chronically infected U1 cells. A crucial mechanism appeared to be the tilting of an endogenous IL-1β/IL-1 receptor antagonist (IL-1ra) balance in favor of the latter (Goletti et al., 1996). In this regard, IL-1ra remains the only natural example of a cytokine receptor antagonist, synthesized in multiple isoforms and expressed both intracellularly and as secreted molecule (Dinarello, 1998), capable of suppressing HIV replication or expression (Kinter et al., 1995b; Muzio et al., 1995; Poli et al., 1994b). It should be underscored that the unique mechanism of IL-1ra is the prevention of IL-1-mediated signaling via interaction with the type I IL-1 receptors, and, therefore, its suppressive effect on virus multiplication is a formal proof of the opposite, enhancing role of IL-1.

As mentioned earlier, IL-16, a physiological ligand of CD4, was early described as being the potential correlate of the nonlytic CD8 antiviral activity discovered in 1986 (Walker et al., 1986). Subsequent studies confirmed that its inhibitory effect on viral transcription and expression (Mackewicz et al., 2000), resembling independent reports describing similar effects induced by some anti-CD4 monoclonal antibodies (mAb) (Briant et al., 1999; Guillerm et al., 1998; Monnet et al., 1999).

CYTOKINES WITH MULTIPLE EFFECTS ON HIV REPLICATION

Most cytokines exert opposite effects on HIV replication in different experimental conditions. IFN-γ, for example, has been reported to inhibit HIV replication at both the entry and post-entry steps (Poli et al., 1994a; Shirazi and Pitha, 1998). Its antiretroviral effect is the result of the activation of the JAK/STAT pathway, following protein tyrosine kinase Pyk2 activation (Takaoka et al., 1999). In addition, we have also described an antiviral effect likely independent from cell surface expression of the IFN-γ receptor 2 chain and involving IFN-stimulated gene factor 3γ (ISGF3γ), a transducer molecule typically activated by IFN-α/β in subclones of the U937 cell line (Bovolenta et al., 1999c). In contrast to these inhibitory effects, IFN-γ activated HIV expression in chronically infected U1 cells (Biswas et al., 1992). Of interest, when U1 cells were activated and differentiated by phorbol esters, IFN-γ appeared to exert a suppressive activity. However, ultrastructural analysis revealed that it actually

redirected the site of virion budding and release from the plasma membrane to intracellular Golgi-derived vacuoles (Biswas et al., 1992). Noteworthy, intracellular accumulation of new progeny virions is a distinctive feature of macrophage infection observed in primary cells infected in vitro (Gartner et al., 1986; Gendelman et al., 1988) as well as in vivo (Orenstein et al., 1997).

In addition to IFN-γ, virtually all the major anti-inflammatory cytokines, namely IL-4, IL-10, and TGF-β, have been associated with either enhancement or suppression of viral replication (Emilie et al., 1992; Kazazi et al., 1994; Lazdins et al., 1991, 1992; Poli et al., 1992; Schuitemaker et al., 1992). In the case of IL-10, HIV replication was inhibited in primary MDM stimulated at concentrations fully suppressing the synthesis of TNF-α and IL-6, whereas exogenous addition of these pro-inflammatory cytokines (at concentrations comparable to those secreted from control MDM) overcame the inhibitory effect of IL-10 (Weissman et al., 1994). Furthermore, when MDM were treated with suboptimal concentrations of IL-10, a moderate enhancement and not inhibition of virus production was observed. IL-10 synergized with both TNF-α and IL-6 in the induction of viral expression in chronically infected U1 cells (Weissman et al., 1995). In addition, IL-10, unlike other anti-inflammatory cytokines, did not diminish virus expression in U1 cells sequentially stimulated with GM-CSF and LPS, a combination leading to the activation of an IL-1β/IL-1ra dependent autocrine loop controlling HIV production (Goletti et al., 1996).

It is likely that several opposite effects on virus replication described for a given cytokine are also consequent to modulatory effects exerted on chemokine entry co-receptors (mostly CCR5 and CXCR4). Indeed, both IL-4 (Schuitemaker et al., 1992) and IL-10 have been shown to favor infection of freshly isolated monocytes (usually more resistant to infection than MDM). In the case of IL-10, this effect was clearly associated to the enhanced expression of CCR5 and CCR5-dependent entry of HIV-1 (Sozzani et al., 1998). A divergent role has been described for IL-4 on the two main entry co-receptors, in that it enhanced CXCR4 expression and decreased CCR5 levels in T cells (Galli et al., 1998; Jourdan et al., 1998; Valentin et al., 1998). In addition, Th1 cytokines such as IL-12 and IFN-γ have also been reported to influence CXCR4 (Shirazi and Pitha, 1998) and CCR5 expression and/or infectability of MDM (Hariharan et al., 1999; Wang et al., 1999) or U937 promonocytic cells (Zella et al., 1998). Thus, all the major cytokines influencing Th1 vs. Th2 immune response show important modulatory effects on HIV entry co-receptors on both macrophages and T cells (Sallusto et al., 1998). It should, however, be underscored that different levels of cell surface expression may not necessarily translate into substantial differences in terms of susceptibility of the target cell to viral infection and spreading. In this regard, we have recently described a "cytokine-like" activity of CCR5-dependent HIV in T cells maintained in IL-2 enriched medium but not mitogenically stimulated by anti-CD3 mAb, a condition that was instead indispensable for the efficient spreading of CXCR4-dependent HIV-1 (Vicenzi et al., 1999). On the other hand, extracellular Tat inhibited entry and

replication of syncytium-inducing (SI) strains but not CCR5-dependent HIV via binding to CXCR4 (Ghezzi et al., 2000). These two last observations provide potential mechanisms through which macrophage-tropic CCR5-dependent viruses dominate SI viruses after transmission and in early HIV disease.

HIV INFECTION PERTURBS CYTOKINE EXPRESSION IN VITRO AND IN VIVO

If several cytokines modulate HIV replication, viral infection or expression of viral proteins can profoundly affect cytokine expression. It was early noted that the levels of pro-inflammatory cytokines are increased in HIV-infected individuals (Fahey et al., 1998; Scott-Algara et al., 1991). In particular, the levels of TNF-α and soluble TNF receptor II are better correlates of disease evolution than $CD4^+$ T cell counts and viremia, respectively (Fahey et al., 1998). In addition, increased levels of anti-inflammatory cytokines, such as IL-10 (Gallo et al., 1994; Muller et al., 1998), and TGF-β (Wahl et al., 1991), have been also observed in vivo and reproduced during in vitro infection (Cota et al., 2000a; McManus et al., 1998). Several viral proteins, such as Env, Tat (Ambrosino et al., 1997; Buonaguro et al., 1994; Ito et al., 1998; Nath et al., 1999), Nef (Collette et al., 1996, 1997; Swingler et al., 1999), or Vpr (Ayyavoo et al., 1997; Poon et al., 1998) have demonstrated multiple effects on cytokine expression. In this context, a major shift from Th1- to Th2-dominant cytokine profiles has been early postulated to play a major role in the pathogenesis of infection, because several exposed but uninfected individuals could elaborate IL-2 secretion or even CTL once their PBMC were challenged ex vivo with HIV peptides (Clerici and Shearer, 1994). However, there has not been a clear-cut body of evidence in favor of this general hypothesis, although Th2-polarized cells are more prone to HIV replication (Maggi et al., 1994). In support of this hypothesis, we have recently observed that primary HIV strains dualtropic for usage of entry coreceptors efficiently replicate in Th2 and Th0, but not Th1 cells (E. Vicenzi et al., unpublished observations).

Clear evidence of the activation of the cytokine network as a consequence of HIV infection has been recently provided by the demonstration of a constitutive activation of STAT proteins in PBMC of infected individuals examined immediately after isolation from peripheral blood. In particular, both STAT1 and STAT5 were detected in the majority (70–80%) of infected individuals, regardless of whether they were or not on antiretroviral therapy (Bovolenta et al., 1999a). In addition, the predominant form of STAT5 was truncated at the C-terminus, therefore maintaining DNA binding capacity but being devoid of biological activity (Bovolenta et al., 1999a); this isoform, in other cell systems, was shown to act as a transdominant mutant for STAT5-dependent gene expression (Bovolenta et al., 1998). Constitutive STAT activation appeared restricted to $CD4^+$ T cells, allowing the speculation that both HIV particles and gp160/120 Env molecules may be involved with this phenomenon (Fig. 14.2).

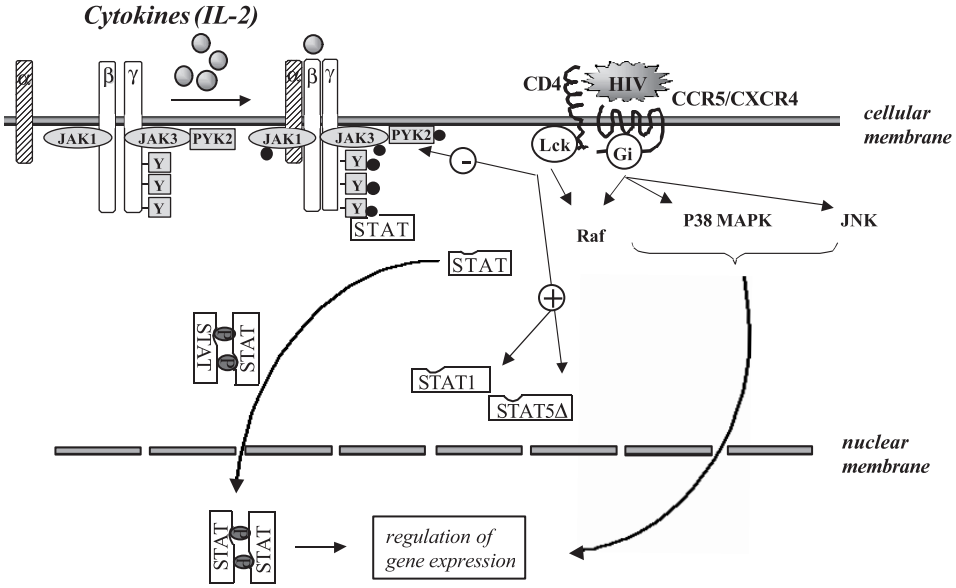

Figure 14.2. Activation of the JAK/STAT pathway in HIV infection. In addition to a variety of cytokines and growth factors that may be dysregulated by HIV infection, either virus replication or viral proteins have been reported to regulate the expression and/or the activation of this important signal trasnduction pathway (Bovolenta et al., 1999a; Pericle et al., 1998). Interaction of the HIV gp120/160 Env with CD4 and/or the chemokine receptor has been shown to lead to the activation of Ca^{++} fluxes and/or focal adhesion kinase Pyk2 (Arthos et al., 2000; Cicala et al., 2000; Davis et al., 1997; Popik and Pitha, 1998; Popik et al., 1998; Selliah and Finkel, 1998; Weissman et al., 1997).

IL-2: A KEY CYTOKINE IN HIV INFECTION

IL-2 deserves a specific place in the study of HIV infection for its multiple effect exerted in vitro, but in particular for its potential of becoming a therapeutic tool for the long-term therapy of infected individuals. IL-2 induces the proliferation and activation of T lymphocytes via heterodimerization of the common β and γ chains of the receptor, whose cytoplasmic tails are critical for transduction of the IL-2 mediated signal, and association of the α chain that increases the affinity of the receptor for the cytokine (Waldmann, 2000). Binding of IL-2 to its own receptor leads to the recruitment and activation of JAK1 and JAK3 to β and γ chains. Transduction of proliferative signal requires JAK3 activity, whereas a JAK3-independent signaling pathway prevents apoptosis and involves various nonreceptor-type tyrosine kinases, such as p56(lck) and other Src family proteins (Waldmann, 2000). However, activation of p56(lck) alone is insufficient for transducing proliferative signals and, therefore, acts in concert with JAK3-mediated receptor activation. In addition, it has been dem-

onstrated that the IL-2 receptor can generate at least three distinct signaling pathways leading to the activation of c-fos/c-jun (AP1), c-myc, and Bcl-2, all essential for IL-2-mediated proliferative signaling. Furthermore, these pathways cooperate with each other to ensure a full-scale cellular activation (Minami et al., 1993).

Concerning HIV infection, it is frequently assumed that IL-2 will stimulate HIV expression, based on its capacity to induce activation and proliferation of T and B lymphocytes. However, IL-2 induces strong cellular proliferation, but, unlike PMA, fails to activate HIV-1 LTR-driven transcription in primary T-cell clones activated in an antigen-restricted manner (Hazan et al., 1990). In addition, IL-2-stimulated PBMC support efficient virus replication that is consequent to the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ acting in an autocrine/paracrine fashion (Kinter et al., 1995b; Vyakarnam et al., 1990). IL-2 was later shown to activate HIV replication (presumably with the same general mechanism) in PBMC and mononuclear cells extracted from lymph nodes of infected individuals and cultivated ex vivo, but unlike IL-12, only when $CD8^+$ T cells were removed (Kinter et al., 1995a, 1996). Thus, IL-2 up-regulated the production of soluble suppressor factors from $CD8^+$ T cells (simultaneously described as dependent from β chemokines or IL-16 by independent groups [Baier et al., 1995; Cocchi et al., 1995]) and natural killer (NK) cells (Oliva et al., 1998) that tilted the balance toward virus suppression. This effect of IL-2 is currently discussed as one of the potential correlates of the in vivo effect of the cytokine once administered to HIV-infected individuals.

Experimental administration of IL-2 was begun in the 1980s after the observation of a relative defect of IL-2 synthesis in infected individuals (Murray et al., 1984). Two main protocols are currently investigated: a low-dose (< 1 MIU/day) delivered continuously per subcutaneous (sc) route (Fehniger et al., 2000; Khatri et al., 1998) vs. administration of intermittent cycles of higher doses of IL-2 (from 3 to 15 MIU/day) given sc for 5 days every 4–8 weeks (Kovacs et al., 1995, 1996). This second approach is currently being evaluated for clinical efficacy in two large-scale international trials (SILCAAT and ESPRIT). The former ''low-dose'' approach is also currently being investigated, and has resulted in substantially increased levels of NK cell activity, without substantial alteration of the representation of PBMC subsets in the periphery. In contrast, the intermittent ''high-dose'' approach leads reproducibly to a very significant increase of circulating $CD4^+$ T-cell numbers reaching normal or near normal levels. Although ''high-dose'' IL-2 administration has resulted in spikes of virus replication in vivo, these were transient in nature and did not alter the set-point of plasma HIV RNA (viremia) (Kovacs et al., 1995), an important prognosticator of disease progression (Mellors et al., 1996). In this regard, we have recently described a down-regulation of Ying/Yang-1 (YY1) and Leader binding protein-1 (LBP-1)/late SV40 factor (LSF), which bind to common sequences in the virus LTR and exert a silencing effect on viral transcription in individuals receiving ''high-dose'' IL-2 therapy (Bovolenta et al., 1999b). As discussed above, IL-2 can also activate HIV-suppressive mecha-

nisms such as those described as "CAF" or chemokines, which may ultimately overcome the capacity of the cytokine to promote virus replication. As for IL-2, other cytokines such as IL-12 and GM-CSF (Angel et al., 2000) are currently being considered for their potential beneficial use in HIV-infected individuals. G-CSF, a molecule devoid of HIV-modulating effects, is broadly used in the clinical settings for fighting the neutropenias often observed in patients with advanced disease.

CONCLUSION

Cytokines have represented an important tool to better understand the profound interactions occurring between HIV and the human immune system. With the discovery of chemokine entry receptors and the related chemokines acting as blockers of HIV infection (as separately discussed in this book), the general paradigm of the cytokine network touching each and every step of the virus life cycle has been formally completed. This general observation is cause for optimism about the possibility that manipulation of the cytokine system will be of substantial help in controlling and, we hope, preventing HIV infection. The fact that at least one cytokine, IL-2, is being currently evaluated in phase III clinical trials in combination with antiviral agents strongly supports this belief.

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PART IV

TECHNOLOGIES

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15

The Use of Peptide/MHC Tetramers to Visualize, Track, and Characterize Class I-Restricted Anti-HIV T-cell Responses

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INTRODUCTION

During the initial burst of immunity to human immunodeficiency virus (HIV)-1 infection, human leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTL) are thought to play an important role in the initial containment of viral load (Borrow et al., 1994). Identification of conserved regions in HIV-1 proteins that are recognized by CTL is important for control of viral replication (da Silva and Hughes, 1998; Goulder et al., 1997b). The important part that CTL play in providing protection is emphasized by their role in long-term nonprogressors (LTNP), i.e., no disease progression (Ogg et al., 1999b), and highly exposed persistently seronegative individuals (HEPS), i.e., protection from infection (Rowland-Jones et al., 1998, 1999). Protective CTL responses have also been shown in experimental viral infection models with lymphocytic choriomeningitis virus (LCMV) (Sourdive et al., 1998; Weidt et al., 1998). The development of CTL responses, identified in HIV-1 clinical studies (Ogg et al., 1999b; Wilson et al., 2000), occurs rapidly in most infected individuals and coincides with the fall in plasma viral load (Wilson et al., 2000), and by this

association, these cells are implicated in controlling the initial burst of viremia. Antiviral pressure from CTL has been demonstrated inferentially in both early (Borrow et al., 1997) and late stages (Goulder et al., 1997a; McMichael and Phillips, 1997) of disease by the degree of variant epitope sequences coinciding with specific CTL responses. Although recent data question the proficiency of immunodominant CTL responses in control of viral replication during chronic HIV-1 infection (Brander et al., 1998), CTL are considered to represent an important arm of protective immunity in the early stages of infection. The thesis that CTL may be ineffective during chronic (asymptomatic) infection will be further explored in this chapter.

Until recently, it was not possible to identify the frequency and phenotypic nature of HLA class I-restricted CTL in the blood circulation. Classical methods for measuring antigen-specific CTL included direct lysis (Dyer et al., 1999), bulk cultures (Lamhamedi-Cherradi et al., 1992), or limiting dilution assays (LDA) (Moss et al., 1995). These methods allow for quantitative (direct lysis or LDA) or qualitative assessment of CTL killing function and rely on either an ex vivo hyperactive state (direct lysis) or in vitro expansion of effector cells (bulk culture or LDA). Although these methods may allow for important functional information to be assessed, they all limit the accurate appraisal of circulating effector cells due to a lack of sensitivity. For example, it has been estimated that to detect a direct anti-HIV CTL response from fresh peripheral blood lymphocytes (peripheral blood mononuclear cells, or PBMC), a frequency of $>1/50$ CTL precursor cells is required to be present in the culture. This is invariably not the case, and bulk cultures or LDA have been successfully used to expand low effector cell frequencies in an antigen-specific manner (Moss et al., 1995). It is highly likely that stimulation-induced cell death occurs during culture (Klenerman and Zinkernagel, 1997) and will result in lower estimations of CTL activity or precursor frequencies. Data reporting the use of peptide/MHC tetramers to measure the frequency of circulating antigen-specific $CD8^+$ cells indicate that the magnitude of anti-HIV-specific T cells is many-fold greater than previously estimated using conventional killing assays (Altman et al., 1996; Murali-Krishna et al., 1998; Whitmire et al., 2000). Furthermore, peptide/MHC tetramer analysis has allowed the direct visualization and phenotypic characterization of antigen-specific T cells (Gray et al., 1999; Ogg et al., 1998).

This chapter will focus on the use of peptide/MHC tetramers and flow cytometry to visualize and measure the frequency of class I-restricted anti-HIV-specific $CD8^+$ cells in the blood circulation of HIV-1-infected individuals. We will also discuss a re-evaluation of the functional role of CTL during viral persistence.

PRINCIPLE OF DETECTION

The most frequently used peptide/MHC tetramers used for HIV studies are made with HLA-A2 folded around immunodominant epitopes (9 mers) in Gag

TABLE 15.1. List of Published Epitopes That Have Been Used to Construct Peptide/MHC Tetramers to Study HIV-1, SIV, EBV, and CMV Infection

Pathogen	Protein Region	Epitope	HLA Allele	Reference
HIV-1	p17 Gag	SLYNTVATL	A*0201	Dyer et al., 1999; Gray et al., 1999; Spiegel et al., 2000a, b; Wilson et al., 1998; Altman et al., 1996; Ogg et al., 1998, 1999
HIV-1	Pol	ILKEPVHGV	A*0201	Dyer et al., 1999; Gray et al., 1999; Spiegel et al., 2000a, b; Wilson et al., 1998; Altman et al., 1996; Ogg et al., 1998, 1999
HIV-1	Nef	FLKEKGGL	B8	Dyer et al., 1999; Spiegel et al., 1999
HIV-1	p17 Gag	GGKKKYKLK	B8	Dyer et al., 1999
HIV-1	Env	DPNPQEVVL	B35	Dyer et al., 1999; Ogg et al., 1999
HIV-1	Pol	EPIVGAETF	B*3501	Dyer et al., 1999
HIV-1	p24 Gag	KRWIILGLNK	B*2705	Wilson et al., 2000
HIV-1	P24 Gag	KRWIIMGLNK	B*2705	Wilson et al., 1998, 2000
EBV	LMP2	CLGGLLTMV	A*0201	Jin et al., 2000
EBV	BMLF1	GLCTLVAML	A*0201	Callan et al., 1998; Jin et al., 2000; Ogg et al., 1999; Tan et al., 1999
EBV	BZLF1	RAKFKQLL	B8	Callan et al., 1998; Tan et al., 1999
EBV	BZLF1	FLRGRAYGL	B8	Callan et al., 1998; Tan et al., 1999
EBV	EBNA3B	IVTDFSVIK	A11	Tan et al., 1999
CMV	pp65	NLVPMVATV	A*0201	Jin et al., 2000; Spiegel et al., 2000b
CMV	pp65	TPRVTGGGAM	B*0702	Gillespie et al., 2000
SIVmac	Gag	CTPYDINQM	Mamu-A*01+	Egan et al., 1999
SIVmac	Pol	STPPLVRLV	Mamu-A*01+	Egan et al., 1999
HIV-1	Env	YAPPISGQI	Mamu-A*01+	Egan et al., 1999

SIV, simian immunodeficiency virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus.

and Pol (Kelleher and Rowland-Jones, 2000) (Table 15.1). Although HLA-A2 is the most common HLA allele in Caucasians, it may not be common in other regions of the world, where the majority of HIV-1 infections are found. Different HLA allele expression by HIV-1-infected people in these regions will determine epitope selection (Gray and Puren, 2000) and will guide the con-

struction of new peptide/MHC tetramers for use in regions where nonsubtype B HIV-1 infections predominate.

Peptide/MHC tetramers were first described by Altman et al. (Altman et al., 1996), who used them to label HIV-1 peptide-specific populations of $CD8^+$ T cells. Syntheses of MHC class I/peptide monomers are created by reacting *Escherichia coli*-expressed MHC class I molecules with a specific viral peptide. The monomers are then biotinylated and made into tetramers with fluorochrome-coupled avidin to form soluble complexes that stably bind to the TCR specific for the viral peptide (Boniface et al., 1998).

Figure 15.1*A* shows a cartoon diagram of a heterogeneous population of $CD8^+$ T cells expressing T-cell receptors (TCR1–4) recognizing a number of HIV-1 epitopes. Only one TCR (TCR 2) can recognize and bind to the epitope presented by the HLA-A2.1 tetramer. Because the core of the peptide/MHC tetramer is avidin, its use after conjugation with biotin-streptavidin-phycoerythrin (PE) will allow TCR-antigen engagement to be detected using fluorescence anlaysis. Thus, staining a population of PBMC consisting of oligoclonally expanded $CD8^+$ cells recognizing HIV-1 epitopes can be rapidly identified using a predetermined peptide/MHC tetramer complex in combination with monoclonal antibodies to CD3 and CD8 surface antigens. HLA class I-restricted epitope responses can then be visualized using standard flow cytometry procedures. Figure 15.1*A* shows a typical three-color staining scenario, with a region (R1) placed around bright $CD8^+$ cells on a forward scatter/CD8-Cy5 plot and gated to a dot-plot of fluorescein isothiocyanate (FITC) (any choice of monoclonal antibody) vs. peptide/MHC PE fluorescence. After collection of 50,000–100,000 events in the $CD8^+$ region, surface expression of an array of different antigens (adhesion molecules or activation markers) may be assessed. In this generic example, 1.2% of $CD8^+$ peptide/MHC tetramer positive cells are dim for CD45RA (Fig. 15.1*A*).

WHAT CAN TETRAMER ANALYSIS TELL US?

The use of peptide/MHC tetramers has allowed a reappraisal of the magnitude of $CD8^+$ T-cell responses to a specified epitope. For example, the magnitude of $CD8^+$ antigen-specific responses to one epitope is much higher than anticipated from killing assays in acute viral infections: LCMV infection in mice (Murali-Krishna et al., 1998) and EBV infection in humans (Callan et al., 1998). It has been shown that 50% of spleen $CD8^+$ cells were specific for LCMV during the acute phase of infection—some 40 times higher than would be accounted for using LDA (Murali-Krishna et al., 1998). It is possible to track ex vivo the frequency of epitope-specific $CD8^+$ cells using a wide range of MHC class I-restricted epitopes during HIV-1 infection. Table 15.1 shows a list of peptide/MHC tetramers that have been used to track antigen-specific responses either in humans or monkeys. The list is not exclusive to HIV-1/SIV and includes EBV

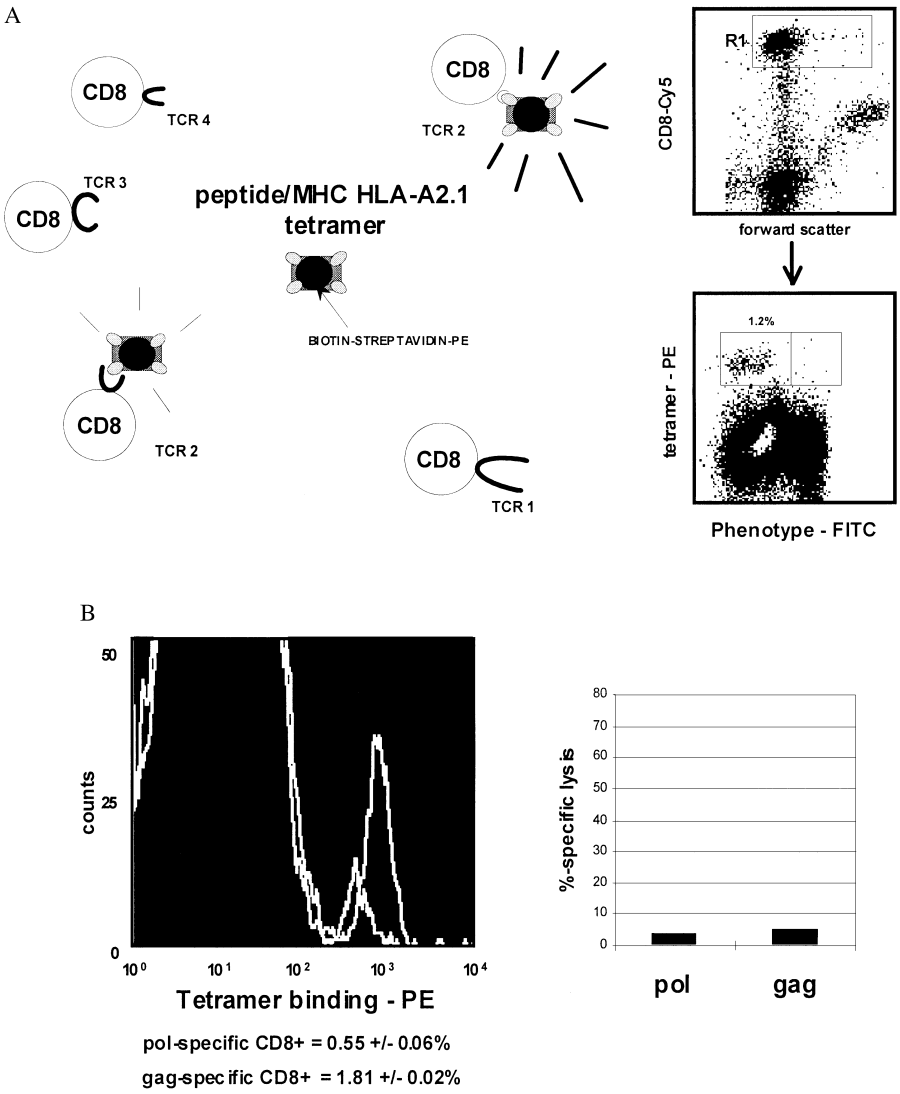

Figure 15.1. (**A**) The principle of peptide/MHC tetramer staining in a mixture of $CD8^+$ T cells expressing different T-cell receptor (TCR) specificities. This example shows an HLA-A*0201 tetramer binding to the TCR recognizing and epitope in Gag. FACS analysis shows gating around high CD8 antigen expression to determine tetramer positivity and surface antigen expression. (**B**) Expression of Gag- and Pol-specific $CD8^+$ cells from fresh PBMC in one representative HIV-1 infected individual with concomitant lack of peptide-specific CTL lysis.

and CMV—allowing the possibility of simultaneously tracking antigen-specific $CD8^+$ T cells to more than one virus within the same individual.

FUNCTION

It may be intuitive to make the extrapolation between quantitative assessments of tetramer-positive cells with functional CTL epitope recognition. However, many studies have noted a discrepancy between tetramer staining and CTL function (Appay et al., 2000; Gray et al., 1999; Zajac et al., 1998), suggesting that these cells are functionally defective. Lack of function (CTL activity; IFN-γ release or perforin content) may be related to loss of $CD4^+$ T-cell help in the case of anti-LCMV $CD8^+$ cells in CD4-deficient mice (Zajac et al., 1998) and HIV-1 infected patients (Spiegel et al., 2000a), due to anergy (Lee et al., 1999), or due to impaired maturation (Appay et al., 2000). It is likely during the asymptomatic stage of HIV-1 infection that tetramer-positive cells are marking either memory cells (Gray et al., 1999) or defective effector CTL. In the acute stage of HIV-1 infection, a recent study has shown that high frequencies of HLA-B27-restricted tetramer-positive cells exist prior to seroconversion and are closely related to the temporal appearance of viral load (Wilson et al., 2000). Moreover, a prior study (Ogg et al., 1999b) shows that tetramer-positive cells during acute HIV-1 infection are probably marking functional CTL that can control viral replication. It may be postulated that at some stage after early HIV infection, antigen-specific cells cease to be functionally competent. This is highlighted by a study that we undertook to examine function of tetramer-positive cells in chronically HIV-1-infected individuals (Gray et al., 1999). Freshly stained tetramer positive cells were readily detectable in the peripheral blood (Fig. 15.1*B*) with Gag-specific cells invariably at higher frequencies than Pol-specific cells. Concomitant direct CTL lysis of PBMC revealed no appreciable CTL activity (Fig. 15.1*B*) (Gray et al., 1999). CTL activity was only observed after exposure of cells to antigen in culture that resulted in a 20- to 40-fold expansion of tetramer-positive $CD8^+$ cells (Fig. 15.2, *A* and *B*).

PHENOTYPE

Flow cytometry offers a valuable tool to simultaneously measure the expression of multiple surface and cytoplasmic antigens and has been used to characterize different cell populations within a heterogeneous mix of PBMC. Peptide/tetramer complexes offer a further tool that allows the phenotypic expression of epitope-specific $CD8^+$ cells.

Early tetramer studies showed that epitope-specific cells were largely to be found in the CD45RA negative population and predominantly expressed CD45RO, compatible with our knowledge of antigen-committed cells losing CD45RA and gaining the CD45RO isoform (Michie et al., 1992). Recent evi-

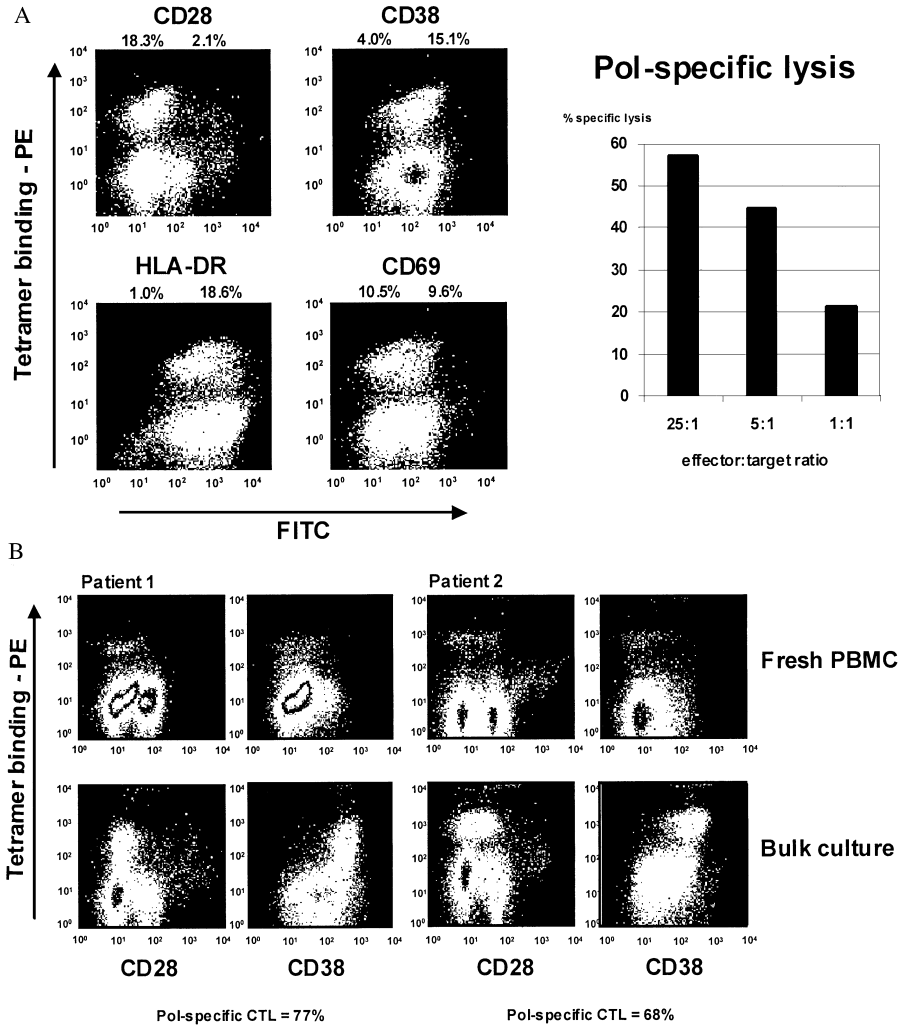

Figure 15.2. (**A**) Expansion of tetramer-positive cells in response to Pol peptide-specific stimulation in bulk culture. There is increased expression of CD38, HLA-DR, and CD69 on expanded antigen-specific $CD8^+$ cells, but lack of CD28 surface expression. These markers were related to high Pol-specific CTL lysis. (**B**) Increased CD38 antigen expression and low CD28 expression in response to Pol-specific stimulus in two other patients.

dence has shown, however, that a small population of CD45RA-expressing cells do express TCR capable of binding peptide/MHC complexes (Gillespie et al., 2000; Lee et al., 1999). Measurement of acute and chronic cell activation expression has opened the question of which surface marker could correlate with effector CTL function. An association of CTL with increased CD38 antigen expression has been made for some time (Mocroft et al., 1997), with evi-

dence shown in this chapter that CD38 expression on tetramer-positive cells may be a marker for functional CTL. We have shown that in vitro antigen stimulation for 14 days causes up-regulation of CD38 antigen expression matching high epitope-specific CTL activity (Fig. 15.2, *A* and *B*). Similarly, there is increased CD69 and HLA-DR expression (Fig. 15.2*A*) and lack of CD28 expression (Fig. 15.2*B*) (Gray et al., 1999). This would be compatible with $CD28^-$ cells being terminally differentiated (Fiorentino et al., 1996; Trimble et al., 2000; Weekes et al., 1999) and being effector cells imparting CTL activity (Gillespie et al., 2000). Interferon (IFN)-γ expression has been associated with CTL function and is the basis of measuring CTL by ELISPOT (Dunbar et al., 1998; Lalvani et al., 1997; Larsson et al., 1999). It would therefore be expected that tetramer-positive cells that express intracytoplasmic IFN-γ would delineate very clearly active CTL from quiescent cells. Different groups have produced variable data as to the extent of tetramer positive cells expressing IFN-γ. We have shown that freshly stained tetramer-positive cells do not constitutively express IFN-γ (Fig. 15.3, *A* and *B*), but only after peptide stimulation (Fig. 15.3*C*). This was found in combination with a large frequency of cells expressing the low-affinity interleukin (IL)-2 receptor (CD25). Other groups have provided compelling data showing IFN-γ production from sorted tetramer-positive cells by Elispot (Ogg et al., 1998). Expression of perforin within tetramer-positive cells may possibly delineate functional effector CTL, although recent data has shown that a patient with melanoma had a large population of $CD8^+$ tyrosinase/tetramer-positive cells, but was deficient in perforin expression (Lee et al., 1999). A further study of HIV-specific and CMV-specific $CD8^+$ cells in chronically HIV-infected individuals revealed that HIV antigen-specific $CD8^+$ cells were impaired in maturation and lacked perforin expression, although they retained the ability to secrete IFN-γ in response to antigen (Appay et al., 2000). Conversely, CMV antigen-specific $CD8^+$ cells expressed significantly larger amounts of perforin and exhibited ex vivo CTL activity (Appay et al., 2000).

TRACKING ANTIGEN-SPECIFIC T CELLS DURING HIV-1 INFECTION

Identifying the dynamics and kinetics of CTL during the course of HIV-1 infection is important in that it provides us with insight into the role of CTL in controlling HIV-1 replication. The influence of HLA class I allele expression within an individual on selection of epitope presentation is crucial to our understanding of whether an epitope is immunodominant. Certain HLA are associated with immunodominance, HLA-B27 and B14, for example (Goulder et al., 1997a), which are also associated with slow progression of disease (Mann et al., 1998). It can be extrapolated that HLA genotypes that are associated with slow disease progression will present immunodominant epitopes found within conserved regions of HIV-1. The resulting CTL responsiveness could then reduce viral antigenic diversity and viral burden (Nowak and Bangham, 1996).

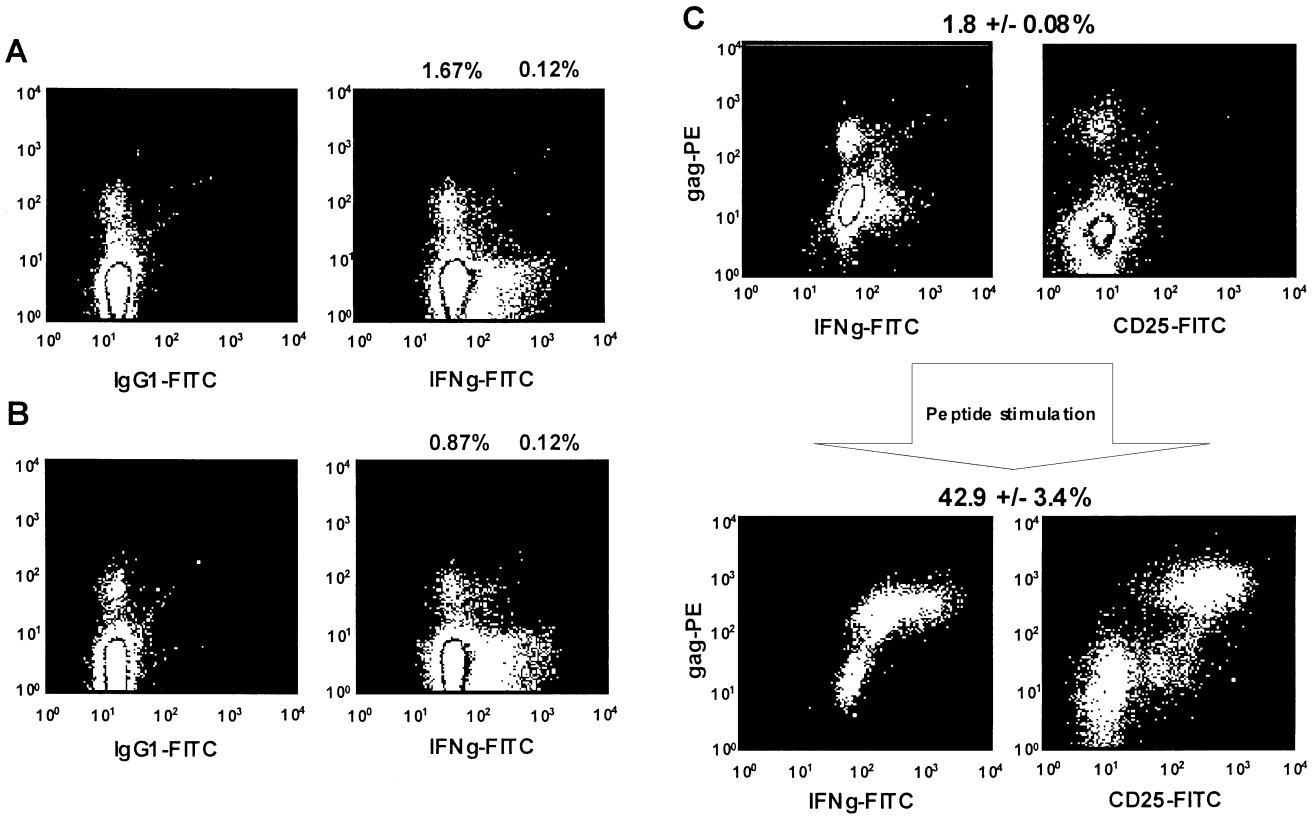

Figure 15.3. (**A and B**) Freshly stained tetramer-positive cells lack constitutive expression of IFN-γ. The first panel set shows the isotype staining control. (**C**) Expansion of tetramer-positive cells in response to Gag peptide stimulation and increased expression of intracytoplasmic IFN-γ and surface expression of CD25 (low-affinity IL-2R). Increased expression is in relation to Gag-specific CTL lysis from 0% in fresh PBMC to a mean of 42.9% after 14 days of culture.

Conversely, disease progression has been associated with HLA homozygosity (Carrington et al., 1999), especially within either the A or B loci (Tang et al., 1999). This may be a result of limited antigen presentation and lack of immunodominance and/or a bias toward Th2 cytokine profiles (Candore et al., 1998). Thus, the choice of peptides for inclusion into a tetramer synthesis is dependent on detailed knowledge of which CTL epitopes are restricted by a particular HLA allele in individuals with a common HLA background. Identification of immunodominant epitopes has been a major thrust of CTL research by many groups and an up-to-date listing and database of epitopes restricted by many HLA alleles is provided by the Los Alamos Immunology Database (Korber et al., 1999). Although this database provides detailed information for subtype B HIV-1 CTL sequences, it does not, as yet, have detailed information of nonsubtype B HIV-1 sequences that may be restricted by HLA alleles found in non-Caucasians. This lack of information will slowly be corrected as more information on non-B subtype CTL sequences will become available from Africa, Asia, and India, where >90% of global infections occur (Karim and Karim, 1999). More importantly, divergent HLA alleles across different geographic regions will influence which CTL epitopes are immunodominant and hence which peptides should be chosen for tetramer synthesis. The importance of tetramer synthesis for tracking antigen-specific cells that recognize non-B subtypes of HIV-1 is important for understanding the course of HIV-1 infection in the context of intercurrent infections (such as *Mycobacterium tuberculosis*) and for evaluating vaccine immunogenicity (see below).

There have been few longitudinal studies that have tracked the course of antigen-specific $CD8^+$ cells during the natural history of HIV-1 infection. The first such study tracked the course of Gag- and Pol-specific tetramer-positive cells in 16 HLA-A*0201 untreated patients over 14 years (Ogg et al., 1999b). High frequencies of Gag- or Pol-specific $CD8^+$ cells at 1–2 years after seroconversion are significantly associated with slow disease progression. Lower anti-HIV and anti-EBV CTL during disease progression was found to be due to preferential loss of the $CD27^+CD45RO^+CD8^+$ cell subset (Ogg et al., 1999b). A subsequent study showed the frequency of Gag (p24) tetramer positive cells in three HLA-B*2705 and −A*0201 positive patients over a 3–4 year period. The immunodominant response appeared to be established prior to seroconversion and tetramer staining reached up to 4.84% of $CD8^+$ T cells that were closely associated with a significant decline in viral load (Wilson et al., 2000).

TRACKING ANTIGEN-SPECIFIC T CELLS DURING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

The impact of HAART on restoration of immunity has been extensively analyzed (Autran et al., 1997; Evans et al., 1998; Gray et al., 1998; Komanduri et al., 1998; Kroon et al., 1998; Li et al., 1998; Oxenius et al., 2000; Roederer,

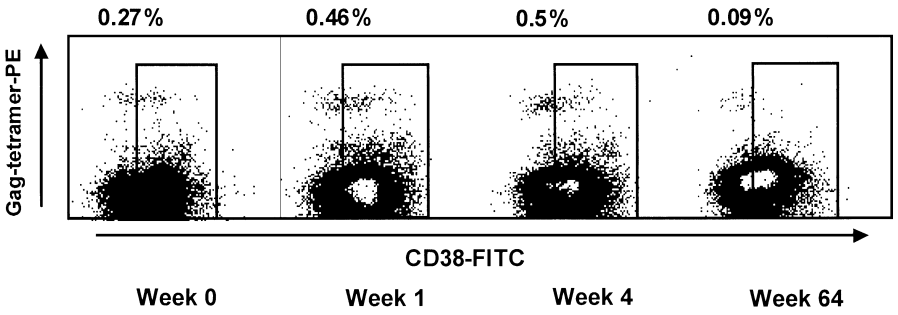

Figure 15.4. Freshly stained $CD38^+$ Gag-specific $CD8^+$ cells in PBMC isolated from an HIV-1-infected patient receiving HAART. An initial increase in the frequency of activated antigen-specific cells was observed in the first 4 weeks of HAART and by 64 weeks was slightly above the detectable threshold on the FACS.

1998) where there are data to show that lymphoid architecture improves (Gray et al., 2000; Haase, 1999; Tenner-Racz et al., 1998; Zhang et al., 1999) and there are increased $CD4^+$ T-cell responses to recall and neoantigens (Valdez et al., 2000). There are also data to show that the frequency of antigen-specific responses in peripheral blood declines in response to HAART (Gray et al., 1999; Kalams et al., 1999; Ogg et al., 1998, 1999a). It has been observed that there is often an initial increase in the frequency of antigen-specific cells in the first 4 weeks after initiation of HAART (Gray et al., 1999; Ogg et al., 1999a), where these cells show increased surface expression of CD38, HLA-DR, and CD69 (Gray et al., 1999) compatible with possible effector CTL function. Figure 15.4 shows a representative patient receiving HAART, where a significant increase in $CD38^+$ Gag-specific $CD8^+$ cells occurs in the first week and thereafter declines (Gray et al., 2000). After 1 year of treatment, the frequency of tetramer-positive cells declined to levels marginally above the lower limits of detection by fluorescence-activated cell sorter (FACS) analysis. It is possible that the initial increased frequency of these activated cells during the early treatment phase reflects release of tissue-resident CTL into the circulation. Other studies have shown that after the initial rise in tetramer positive staining, a steady decay of tetramer-positive cells (median half-life of 45 days) continues for as long as there is suppression of HIV replication (Ogg et al., 1999a). Collectively, these data suggest that suppression of high levels of replicating HIV-1 during the chronic stage of infection removes the stimulus required for maintaining HIV-specific $CD8^+$ T cells. Hence, it is unlikely that HIV antigen-specific cells during chronic infection are controlling viral replication. Conversely, recent data have shown that provision of HAART at the time of seroconversion results in maintenance of numbers and function of $CD4^+$ and $CD8^+$ antigen-specific T cells (Oxenius et al., 2000).

ROLE OF TETRAMER STAINING FOR MONITORING VACCINE IMMUNOGENICITY

The use of peptide/MHC tetramers for rapidly assessing the cellular immunogenicity of therapeutic or preventative vaccine candidates represents an important development. Peptide/MHC tetramers have been used to monitor vaccine responses in rhesus monkeys with a Mamu-A*01 MHC background (Letvin et al., 1999). Antigen-specific $CD8^+$ T cells were first identified in SIVmac 251 infected animals using tetramers folded around the immunodominant epitope in Gag: p11C.C-M (Egan et al., 1999). These tetramers were then used to assess the magnitude of the CTL response elicited by modified vaccinia Ankara (MVA) expressing the SIV *gag* and *pol* genes (Seth et al., 1998). Four of four Mamu-A*01 positive animals possessed 0.6–4.9% p11C.C-M/Mamu-A*01 tetramer binding cells 10 days after a second immunization with MVA (Seth et al., 1998). Similar results have been shown in monkeys receiving a DNA prime followed by an MVA boost (Hanke et al., 1999). Use of peptide/MHC tetramers in clinical vaccine trials has yet to be reported.

CONCLUSION

This chapter has shown that flow cytometry coupled with peptide/MHC tetramers can be used to measure the magnitude of MHC class I-restricted T-cell responses directly in the peripheral circulation. The method is quick, and the frequency of antigen-specific cells can be quantified without the requirement for labor-intensive culture conditions. The nature of HIV infection, as persistent and chronic, results in clonally expanded numbers of antigen-specific $CD8^+$ T cells. The use of peptide/MHC tetramers has shown that during the acute stage of infection, large numbers of antigen-specific $CD8^+$ cells are functionally active and are associated with control of HIV-1 replication (Ogg et al., 1999b; Wilson et al., 2000). However, during the chronic stage of infection, in the clinically asymptomatic stage, large numbers of tetramer-binding $CD8^+$ cells are not functional and probably require persistently high levels of HIV-1 replication to maintain their numbers (Gray et al., 1999; Kelleher and Rowland-Jones, 2000). This scenario has been gleaned from studies showing that HAART results in significant reductions in the frequencies of antigen-specific cells as HIV-1 replication is suppressed. The hypofunctional nature of tetramer-binding $CD8^+$ T cells has also been found in studies investigating melanoma-specific T cells (Lee et al., 1999) and in CD4-deficient mice infected with LCMV (Zajac et al., 1998). It is tempting to equate the progressive loss of CD4 help with loss of anti-HIV CD8 CTL function as HIV-1 disease progresses. A recent study has shown that lack of perforin content and CTL killing capacity during the chronic stage of disease may be related to a block in $CD8^+$ CTL maturity (Appay et al., 2000). Whatever the reason for defective antigen-specific $CD8^+$ T cells after initial viral infection, the use of peptide/MHC tetramers has

allowed us to re-evaluate the magnitude and functional nature of $CD8^+$ T cells during the different stages of HIV-1 infection. Importantly, this evaluation has allowed us to rethink therapeutic strategies and find mechanisms for switching on defective antigen-specific $CD8^+$ T cells and mobilize their anti-HIV CTL activity during chronic disease.

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16

Tagging of HIV with Green Fluorescent Protein

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INTRODUCTION

Starting in 1995, the use of the green fluorescent protein (GFP) in retroviral research has been constantly growing. GFP is now used for the detection of infected cells, studying viral trafficking, and characterization of viral proteins functions, and as a reporter in the development of the gene therapy approaches. GFP has been used as a label in studies of human immunodeficiency virus (HIV) in at least four methodologically different ways. Two approaches were used for the production of fluorescent virions, labeling *in cis* and *in trans*. For labeling *in cis*, GFP is incorporated into the viral genome. The recombinant viruses express GFP during replication, and, thus, infected cells can be identified easily with fluorescence detection methods. Labeling of viral particles *in trans* takes place during self-assembly of the virions. One of the viral proteins is coexpressed as a fusion with GFP simultaneously with the virus and is spontaneously and very specifically incorporated into the viral particles. The resulting fluorescent virions allow for direct observation of one cycle of virus infection, but they do not replicate as fluorescent particles. GFP is also widely used as a reporter gene for fluorescence-based detection of retroviral infection and for measurement of gene transfer with HIV-derived retroviral vectors. Fusion of many HIV-1 proteins to GFP was used for studying the function and trafficking

of the proteins. All these approaches provide beneficial tools with a variety of applications and thus will be described in the present chapter.

LABELING OF HIV-1 VIRIONS *IN CIS*

Immunohistochemistry has been widely used for detection of HIV-1-infected cells both in peripheral blood mononuclear cells (PBMCs) of patients with acquired immunodeficiency syndrome (AIDS) and in cultured cells (reviewed by McSharry, 2000). HIV-1 recombinants producing GFP allow for much more rapid and easy detection of virus-infected cells by fluorescent microscopy, fluorescence-activated cell sorter (FACS) analysis, and multi-well fluorescence reading. Two groups have reported generation of replication-competent GFP-tagged HIV-1 (Lee et al., 1997; Page et al., 1997). In both cases, the *nef* gene in the molecular clone of HIV-1 was replaced with the gene encoding GFP. A total of 293 cells were transfected with the plasmid-encoding GFP-tagged HIV-1, and the labeled virions were harvested from the supernatant and used for infecting H9, a human leukemia cell line (Page et al., 1997). Page and colleagues have found that virus labeled with wild-type GFP did not produce sufficient fluorescence in infected cells, whereas S65A mutant generated 10- to 20-fold greater fluorescence intensity, making it applicable for single-cell analysis under standard conditions of flow cytometry. Both groups have found that insertion of the GFP gene did not affect viral replication. Lee and co-workers have analyzed the genomic stability of the recombinant GFP-tagged virus and found no significant reduction in the expression of viral and GFP genes, even after 10 passages. GFP-tagged HIV-1 was suggested to be a useful tool in studying the biology of HIV replication in vitro and in animal models and for screening of anti-HIV-1 drugs. The HXB-2D clone of HIV-1 with the enhanced GFP gene inserted in place of the *nef* reading frame was used to examine the dependence of HIV-1 on its nuclear factor (NF)-κB binding sites within the long terminal repeats (LTR) of the HIV-1 genome (Chen et al., 1997). Comparison of wild-type HIV-1 with the virus mutated in the κB site of LTR allowed for the demonstration that in T cells, the virus can use the transcription factor NF-κB to enhance its growth but is also able to grow in its absence.

Application of a virus labeled with GFP *in cis* allowed easy analysis of viral gene expression pattern in a single cell. GFP tagging has several advantages over other methods of detection of viral infection in vitro. First, the infected cells can be identified without any additional processing steps. Second, GFP allows for identification of live infected cells without any damage to them. Third, virus-infected cells can be easily separated by FACS analysis.

Similar to HIV-1, simian immunodeficiency virus (SIV) was engineered to express enhanced GFP *in cis* (Alexander et al., 1999). Unlike HIV-1, labeled SIV can be used not only for in vitro, but also for in vivo studies. Interestingly, replacement of the *vpr* sequence in SIV with EGFP resulted in a vector that caused transfected cells to fluoresce but did not produce replication-competent

viruses. The authors suggest that GFP gene insertion may interfere with the appropriate splicing or expression of other viral products. Recombinant virus with GFP inserted into the *nef* locus replicated with wild-type kinetics and produced brightly fluorescent cells, similar to HIV-1. SIV Δnef EGFP plasmid produced infectious SIV. A related plasmid with the HIV-1 *nef* sequence expressed by downstream placement of an internal ribosomal entry site produced similar results. The strains were infectious in rhesus monkey, and infected cells could be detected in the tissue of infected animals by FACS analysis and fluorescence microscopy. The GFP gene was found to be expressed through several rounds of viral replication in cultured cells. However, GFP expression in vivo turned out to be much less stable. Seven days after inoculation, all SIV yielded from PBMCs were fluorescent, whereas $<10\%$ of the virus recovered at day 21 had detectable fluorescence. By day 35, none of the recovered SIV caused cells to fluoresce. Analysis of polymerase chain reaction (PCR)-amplified regions of SIV genome from genomic DNA isolated from PBMCs of infected animals revealed deletions within the GFP sequence. Thus, SIV recombinants expressing GFP were suggested as a tool in studying early events in SIV infection. The instability of the genomes in vivo hampers application of GFP-tagged SIV for long-term studies of virus-host interaction.

LABELING OF HIV-1 VIRIONS *IN TRANS*

Fluorescent viral particles allow for the microscopic studies of virus trafficking, which is important for understanding the mechanisms of viral entry. The attachment of the virus to the cells and the subsequent entry steps leading to infection have become popular targets in the development of novel antiviral agents. The potential of the development of potent antiviral agents stimulated the studies of mechanisms of viral attachments and the pathways of the virions' trafficking. Electron microscopy still remains the major method to visualize the interactions between a virus and the cell, but it does not allow for observation of viral trafficking in real time in live cells. It is also very laborious, especially when numerous cells have to be examined; and the harsh fixation procedures can give rise to artifacts. In the past, chemical modifications of assembled virus particles and intercalation of fluorescent lipids into the particle envelopes were used for labeling of virions. Greber and colleagues successfully studied the nuclear entry of adenovirus labeled with fluorescein isothiocyanate (FITC) (Greber et al., 1997). Nichols and co-workers have used FITC-labeled influenza virus and flow cytometry to assess binding and internalization of virions by monocytes-macrophages and lymphocytes (Nichols et al., 1993). The vesicular stomatitis virus labeled with FITC enabled observation of endocytosis and intracellular trafficking of the virus to the nucleus of infected cells (Da Poian et al., 1996). Kinetics of intracellular disassembly and processing of the vesicular stomatitis virus were probed by Bodipy fluorescence dequenching (Da Poian et al., 1998). The virus was covalently labeled with the fluorescent probe Bodipy-

FL, and the resulting conjugate was 97.6% self-quenched due to fluorescence resonance energy transfer between neighboring Bodipy molecules. Fluorescence microscopy and flow cytometry experiments with cells incubated with Bodipy-VSV revealed intracellular relaxation of fluorescence self-quenching resulted from viral disassembly and viral protein degradation.

The large size and the high protein content of the adenovirus and VSV particles facilitate specific conjugation with fluorescent dyes and detection, but may not be applicable to other viral systems. The approach did not prove to be efficient in the case of HIV-1 and was complicated by extensive labeling of contaminating microvesicles that are always present, even in the purest viral preparations (Bess et al., 1997; Gluschankof et al., 1997). Incorporation of fluorescent components during self-assembly of viral particles allows for the most specific labeling of the virions. Virions of potato virus X (PVX) were the first ones to be successfully labeled with the GFP (Cruz et al., 1996). GFP was introduced as a fusion with the coat protein within the viral genome. However, assembly of fluorescent virions required the presence of free coat protein in addition to the fusion protein subunits. Fluorescent virions could be imaged in infected plant tissue using confocal laser scanning microscopy. It is noteworthy that the resulting virions had an altered morphology, being twice as large as wild-type virions. Thus, GFP attachment influences PVX coat protein oligomerization and virion assembly.

For sufficient intensity of fluorescence, the protein that is chosen for labeling of viral particles during self-assembly should be incorporated in virions in multiple copies. However, modification of major structural viral proteins with a label of a comparable size is likely to interfere with correct assembly of viral core or coat. In case of HIV-1, accessory proteins seem to be good candidates for labeling of whole virions. Among them, HIV-1 viral protein R (Vpr) appears to be the optimal choice because it is incorporated into virions in relatively large quantities. Vpr is a 96-amino acid protein that is involved in the nuclear import of viral DNA and the growth arrest of host cells (Bukrinsky and Adzhubei, 1999). Recent studies have demonstrated that Vpr is present in viral particles in a molar ratio of approximately 1:7 compared with capsid (Muller et al., 2000). However, the extent of incorporation of HIV-1 Vpr into the virus particles is flexible and can be modulated by expression level in cells (Lai et al., 2000). Vpr was suggested as a carrier for targeting foreign proteins into primate lentiviruses (Park and Sodroski, 1996). It was successfully used for incorporation of chloramphenicol acetyltransferase (CAT) enzyme into SIV (Park and Sodroski, 1996) and HIV-1 (Yao et al., 1999). An anti-integrase single-chain variable fragment moiety delivered into viral particles by fusing it to Vpr was used for inactivation of HIV-1 virions (BouHamdan et al., 2000; Okui et al., 2000).

Vpr fused to the C-terminus of GFP incorporated efficiently *in trans* during HIV-1 assembly and produced highly fluorescent virions (Stauber et al., 1999). GFP-labeled virions were prepared by cotransfection of 293 cells with HIV-1 molecular clones and a plasmid expressing highly fluorescent variant of GFP

(Stauber et al., 1998a) fused to the N-terminus of Vpr under the control of the cytomegalovirus promoter (Stauber et al., 1999). Two and a half-fold excess of the GFP-Vpr expression plasmid was used in cotransfections to achieve efficient incorporation of the tagged Vpr over the Vpr encoded by the molecular HIV-1 clones. GFP-Vpr-labeled HIV-1 virions were infectious and replicated as efficiently as unlabeled control virus preparations in human T-cell line. They also displayed normal size and morphology when examined by electron microscopy.

The fluorescent signal generated by the incorporated GFP-Vpr was very intense and could be easily detected by confocal laser scanning microscopy (CLSM) (Fig. 16.1). The resulting fluorescent virus particles were used for studying the mechanisms and kinetics of viral entry and the roles of discrete cell surface receptors, CD4, and chemokine receptors in various cell lines (Stauber et al., 1999). The approach, in contrast to electron microscopy, allowed continuous monitoring of virus entry in living cells. We have used it for direct visualization of HIV-1 attachment and trafficking in various cell lines. Safety considerations required inactivation of HIV-1 by mild fixation before microscopic observation in this study. Confocal microscopy analysis using GFP-Vpr virions in combination with fluorescent endosomal markers showed that, even in macrophages, only a small portion of virus particles enter cells through endocytosis. Direct fusion with cellular membrane represented the major route of entry. Intracellular-labeled virions were observed within minutes after infection and migrated further into the cytoplasm. The movement from the plasma membrane to the perinuclear area could be followed for at least 60 min after infection. However, we did not detect significant accumulation of viral particles at the nuclear membrane or in the nucleus. GFP signal disappeared approximately 90 min after infection, presumably due to the disintegration of the virus core resulting in dilution of GFP-Vpr to a concentration no longer detectable by CLSM. A more sensitive detection system and an opportunity to conduct the study without fixation that causes partial quenching of GFP fluorescence may allow for longer observation of trafficking of the preintegration complexes.

The generation of HIV-1 virions labeled with GFP-Vpr *in trans* is a relatively straightforward procedure and thus can be easily applied to many different molecular clones of the virus. We have obtained two labeled HIV-1 strains, NL4-3 and JR-CSF, and have observed significant differences between the two in dependence of entry on the expression of the chemokine receptors. Visualizing HIV-1 attachment and entry in the absence or presence of CD4 and/or the appropriate coreceptors indicated that CD4 is the major receptor for virus attachment in the case of JR-CSF and NL-4-3 HIV-1 isolates, however, the coreceptors are required for membrane fusion. Internalization of the coreceptor CXCR4 inhibited entry of NL-4-3 HIV-1 isolate, but did not prevent virus binding. Application of GFP-Vpr virions allowed for a visual analysis of the mechanisms and molecular determinants of viral entry. An experimental strategy that involves autofluorescent virions in combination with various fluorescent probes may be applied to many viral systems in living cells to dissect the dynamics of virus entry and the contribution of cellular receptors.

Fluorescent HIV-1 virions were also generated with the help of Vpr fused to GFP through the C-terminus of Vpr (Muthumani et al., 2000). Vpr-GFP exhibited the same activity in arresting the cell cycle in G_2 phase as the wild-type protein and replication kinetics showed no significant differences between Vpr-GFP and native complemented pseudovirus. In this study, amphotropic pseudotype viruses lacking *vpr* were used. Production of fluorescent virions was accomplished by cotransfection of human rhabdosarcoma (RD) cells with three constructs encoding envelope protein, viral genome lacking *vpr* and *env*, and Vpr-GFP plasmid. The resulting particles were tested for the ability to infect target cells and deliver detectable amounts of GFP. Twenty-four hours post-infection, GFP-labeled virus was seen to have infected normal human PBMCs, macrophages, and dendritic cells. The infected cells were fluorescent and could be identified by fluorescence microscopy and FACS analysis. GFP fluorescence exhibited perinuclear localization in infected cells. The study strongly suggests that Vpr-GFP fusion protein can be used to identify subsets of important cells infected in a complex cell background.

GFP AS A TRANSCRIPTIONAL REPORTER FOR HIV-1 LTR

Transcriptional activation of reporter genes is a widely used approach in the development of highly sensitive detection systems. LTR are promoters that are the hallmarks of the retroviruses. DNA sequences within LTR interact with a variety of cellular factors (reviewed in Pereira et al., 2000). Tat protein encoded by the virus is a potent transactivator for HIV gene expression. It binds to the TAR region within the LTR. A number of HIV-1 detection systems have been developed that use a reporter gene (luciferase, β-galactosidase, alkaline phosphatase, CAT) under the control of HIV-1 LTR. The cells of interest are initially transfected with the reporter plasmid containing HIV-1 LTR upstream from the marker gene. Upon infection with HIV-1, the viral *tat* product increases transcription from LTR promoter, leading to high level of expression of the reporter protein. Unlike previously used reporter systems, GFP-based detection does not require processing of the cells by fixation, lysis, or incubation with a substrate. HIV-1 infected cells can be detected with high sensitivity by either fluorescence microscopy or FACS analysis. Originally, the system was introduced by Dorsky and colleagues (Dorsky et al., 1996) as a tool for titering HIV-1 and sorting HIV-1 infected cells. The authors have modified the commercial plasmid-expressing S65T mutant of GFP (pS65T-C1) from Clontech by substitution of the HCMV IE promoter with HIV-1 LTR. HeLa-CD4 cells transfected with the plasmid became brightly fluorescent when infected by HIV-1 or after cotransfection with *tat*-expressing vector. The background fluorescence of the cells carrying only marker plasmid was very weak.

Transcriptional activity of the LTR of four different HIV-1 primary isolates was compared with the help of a transient expression system utilizing LTR-GFP vectors (Kar-Roy et al., 2000). The comparison conducted in HeLa cells

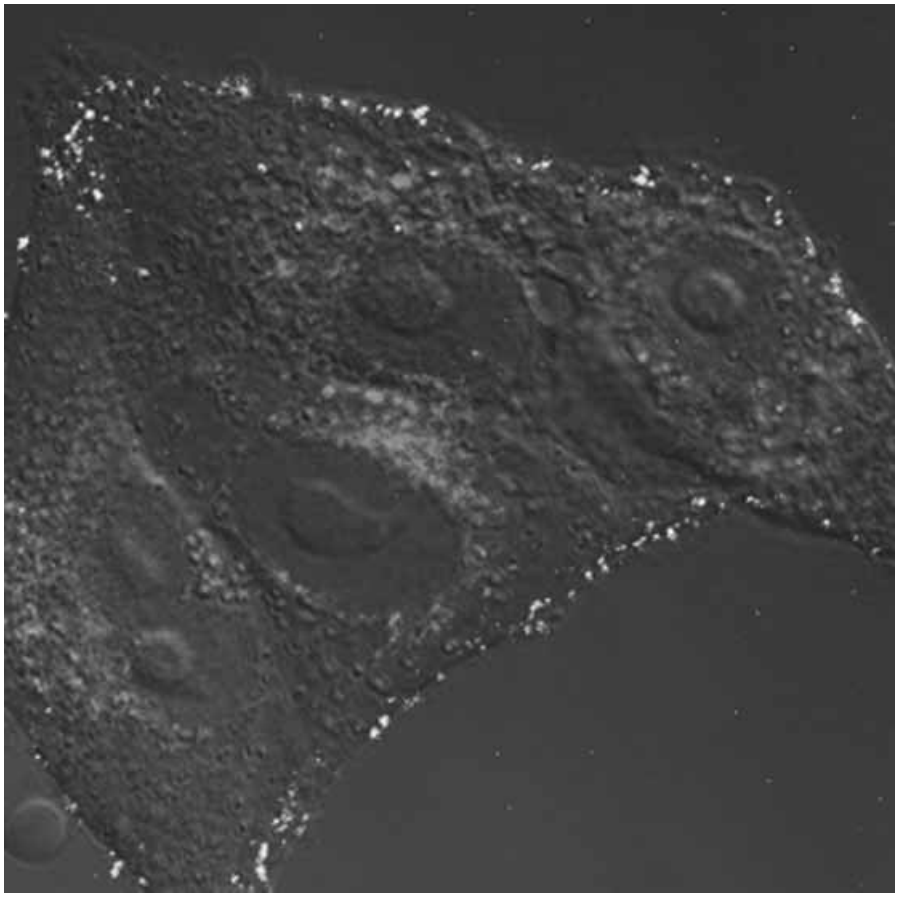

Figure 16.1. Confocal laser scanning microscopy of $CD4^+$ HeLa cells incubated with GFP-tagged HIV-1 virions that are seen as dots in the periphery of the cells. The endosomal compartments are labeled with rhodamine red transferrin and appear mostly in the perinuclear area of the cells. The cells were fixed 10 min after application of the virus. The size of the spots corresponding to the labeled virions does not reflect the real size of viral particles. Virions appear much larger due to the very strong fluorescent signal of GFP resulting in scattering of photons in microscope detector. No colocalization of virions with endosomes could be detected.

revealed significant differences in responses to Tat between different LTRs. The authors have found the assay to be accurate and sensitive, but only in a certain interval of amount of DNA used for transfection. The problem can be alleviated and the test can be made easier and more reliable by the generation of stably transfected cell lines. A stable T-cell line (CEM) expressing a plasmid-encoding humanized enhanced GFP under the control of HIV-1 LTR was shown to respond to HIV-1 infection by a 100- to 1000-fold increase in fluorescence (Gervaix et al., 1997). The activity of antiretroviral drugs with different mechanisms of action was evaluated in CEM-GFP cells. All compounds

tested (the reverse transcriptase inhibitors zidovudine and nevirapine, the protease inhibitor saquinavir, the gag transport inhibitor cyclophilin, and the tat inhibitor Ro24-7429) produced IC50 values that were equivalent to a standard assay using p24 antigen reduction. The HIV-1 LTR can be activated by a number of cellular proteins (Pereira et al., 2000). However, upon stimulation with the most potent cellular stimulants, such as tumor necrosis factor (TNF)-α and 12-myristate 13-acetate, the fluorescence of CEM-GFP cells increased 2- to 3-fold compared with over 100-fold with HIV-1 infection (Gervaix et al., 1997). Psoralen-UV light-inactivated virus, which is able to bind and enter the cells but unable to replicate, produced no GFP in inoculated CEM-GFP cells. CEM-GFP cells were used to demonstrate Nef-induced down-regulation of major histocompatibility complex class 1 (MHC-1) (Akari et al., 2000). Immunostaining of the cell-surface MHC-1 in infected cells followed by FACS analysis revealed that fluorescence intensity for GFP inversely correlated with MHC-1 expression for the wild-type NL-432 HIV-1 isolate. In contrast, infection with Nef-defective mutants was not accompanied by MHC-1 down-regulation. The CEM-GFP reporter system seems to be a valuable tool that can be applied to high-throughput assays for anti-HIV-1 drugs and rapid determination of viral infectivity titers. However, CEM that can be infected with T-cell-tropic HIV-1 isolates lack CCR5 receptor that is mandatory for macrophage-tropic or non-scyncytium-inducing (NSI) isolates of HIV-1. Engineering the cells that express a variety of coreceptors used by HIV-1 for cell entry will make the assay applicable for broad screening for anti-HIV-1 agents. The method is rapid, easy and inexpensive. The results can be read by flow cytometry, cytofluorometry, or fluorescent microscopy.

Human osteosarcoma (HOS) cells stably transfected with a reporter construct consisting of the HIV-2 LTR upstream of humanized GFP was used to generate a panel of cell lines (GHOST cells) expressing CD4 and one of eight chemokine receptors that mediate HIV-1 entry into cells (Cecilia et al., 1998). The system allowed for testing of a large number of primary HIV-1 isolates for their coreceptor preference. Coreceptor usage was discerned for each virus by measuring the number of fluorescent cells by flow cytometry in preparations of infected cells. The assay was also used to study the parameters that contribute to the sensitivity of HIV-1 isolates to neutralization by human monoclonal and polyclonal antibodies (Cecilia et al., 1998). GHOST cells are now available through the National Institutes of Health AIDS Research and Reference Reagent Program (URL available at *http://www.aidsreagent.org*) due to the generous donation of Dr. Littman, and they are becoming a popular tool in studying inhibition of HIV-1 replication. They were used to investigate whether the identity of the coreceptor used by primary HIV-1 isolates influences the sensitivity of these isolates to neutralization by monoclonal antibodies and CD4-reagents. These studies showed that neutralizing antibodies are unlikely to be the major selection pressure in the evolution of HIV-1 coreceptor usage (Trkola et al., 1998). GHOST cells were used to characterize the phenotype of SIV viruses that emerge in immunization with attenuated strains of the virus

(Sodora et al., 1999). GHOST cells proved to be instrumental in a high-throughput screening assay of neutralizing activity of sera from the patients who are long-term nonprogressors and were receiving highly active antiretroviral therapy (HAART) (Dreyer et al., 1999).

Murine cell line NIH 3T3, which stably expresses a reporter of HIV-1 infection, the LTR-GFP cassette, was used to define molecular determinants for HIV-1 infection (Mariani et al., 2000). Infection and replication were assessed by flow cytometry. Stable expression of human CD4, chemokine receptor CCR5, and cyclin T1 allowed an M-tropic virus to enter efficiently, form provirus, and be expressed. However, the virus failed to replicate, presumably because of a major block at virion assembly. A murine model for HIV-1 replication would allow investigation of features of AIDS such as the immune response to the virus, the dynamics of virus replication, and the mechanism of T-cell depletion. The suggested LTR-GFP reporter system can assist in determining all factors that are needed for the development of such a model.

LABELING OF VIRAL PROTEINS WITH GFP

Intracellular protein trafficking is an important component of function that can regulate protein activity and determine involvement in certain processes. GFP has been used widely as a unique tool to monitor dynamic processes in living cells (reviewed by Whitaker, 2000) and it was used extensively for studying the function of HIV proteins. HIV-1 encodes 15 distinct proteins (Frankel and Young, 1998). Substantial progress has been made toward understanding the function of each protein, the Gag and Env structural proteins MA (matrix), CA (capsid), NC (nucleocapsid), p6, gp120 (surface envelope), and gp41 (transmembrane envelope); the Pol enzymes PR (protease), RT (reverse transcriptase), and IN (integrase); the gene regulatory proteins Tat and Rev; and the accessory proteins Nef, Vif, Vpr, and Vpu.

Rev

The Rev protein was the first HIV-1 protein to be fused to GFP (Stauber et al., 1995). Rev activates export of unspliced viral RNA from the nucleus (Pollard and Malim, 1998). It shuttles between the nucleus and the cytoplasm and harbors both a nuclear localization signal and a nuclear export signal. Therefore, HIV-1 Rev represents an excellent system for studying the transport across the nuclear envelope. Fusion to the C-terminus of GFP did not affect the biological activity of Rev (Stauber et al., 1995). A GFP-fusion protein was used to study the mechanisms of Rev inactivation by mutant Rev proteins displaying a transdominant phenotype. Upon actinomycin D treatment, Rev-GFP was transported to the cytoplasm, whereas transdominant Rev(TDRev), although partially dissociated from the nucleolus, was retained in the nucleus. Coexpression of Rev-GFP and TDRev in the same cell demonstrated that TDRev inhibited

the transport of Rev-GFP from the nucleus to the cytoplasm. This inhibition was specific for Rev, because the export of the functionally analogous Rex protein of the human T-cell leukemia virus type I was not inhibited by TDRev. These studies have suggested that Rev and TDRev form heteromultimers in the nucleolus and that this interaction prevents Rev's export from the nucleus to the cytoplasm. Rev mutants that are retained in the cytoplasm were also labeled with GFP (Stauber et al., 1998b). Application of dual-color GFP tagging revealed that coexpression of the nucleolar blue-tagged WT Rev protein together with green-labeled cytoplasmic Rev mutants did not result in the retention of WT Rev in the cytoplasm but, on the contrary, in colocalization of the mutants to the nucleolus. The cytoplasmic mutants were not transdominant and thus are not suitable for Rev inhibition. Rev-GFP fusion protein was used in studies of the protein function in human astrocytes (Ludwig et al., 1999). Astrocytes are known to harbor the virus in a nonproductive manner, and structural HIV-1 proteins that depend on Rev in their expression were very rarely detected in this type of cell. In all astrocytic cell cultures, Rev-GFP was found in both cytoplasmic and nuclear compartments, in contrast to the typical nuclear/nucleolar localization in HIV-1-permissive control cells. The authors have suggested that diminished Rev response in astrocytes is associated with cytoplasmic localization of Rev.

To study nuclear export of Rev in isolation, investigators generated a GFP-labeled, hormone-inducible Rev chimeric protein (Love et al., 1998). The sequence encoding full-length Rev and the hormone-responsive element from the rat glucocorticoid receptor (GR) was inserted into pGreenLantern from Life Technologies (Baltimore, MD). Rev/Gr/GFP behaved like wild-type Rev in living cells. Steroid removal switches off import, allowing direct visualization of the Rev export pathway in living cells. Studies have demonstrated that a functional nuclear export sequence (NES), adenosine triphosplate (ATP), and fractionated cytosol were sufficient for nuclear export in vitro. Nuclear pore-specific lectins and leptomycin B were potent export inhibitors. Nuclear export was not inhibited by antagonists of calcium metabolism that block nuclear import. The distinct requirements for nuclear import and export suggest that these competing processes may be regulated independently.

Rev-GFP construct was used for the comparison of ten Rev-type nuclear export sequences found in different proteins and for structure-activity studies of NES (Henderson and Eleftheriou, 2000). NES were found to vary profoundly in activity and were inactivated by mutation of key hydrophobic residues. GFP has proved to be instrumental in studying Rev trafficking between nucleus and cytoplasm and thus can be used for further detailed analysis of nuclear import and export.

Tat

Fusion of HIV-1 transactivator protein to GFP was used to study intracellular localization, trafficking, and interactions of Tat in human cells (Stauber and

Pavlakis, 1998). Fusion of the C-terminus of Tat to GFP had no effect on the transactivating activity of the protein. When expressed at relatively low levels, Tat-GFP was found in the nucleus, whereas overexpression resulted in nucleolar accumulation. A Tat-GFP hybrid containing the Rev nuclear export signal localized mostly in the cytoplasm. Nevertheless, it was able to transactivate HIV-1 LTR, suggesting that even transient presence of Tat in the nucleus is sufficient for activity.

Vpr

Vpr fusions with GFP were used for labeling of HIV-1 virions *in trans* (Muthumani et al., 2000; Stauber et al., 1999) When expressed in cells, both Vpr-GFP and GFP-Vpr had nuclear localization that is characteristic for nonlabeled protein. Fusion to GFP was applied for identification of the Vpr domains that are involved in nuclear localization (Kamata and Aida, 2000). The first predicted N-terminal α-helical domain (αH1) and the second helical domain located close to the C-terminus (αH2) were fused individually with GFP. Both constructs were localized predominantly in the nucleus. The nuclear localization pattern was eliminated by mutations in the helixes, suggesting that the two domains are involved in nuclear import of Vpr by certain cellular factors.

Integrase

Karyophilic properties of HIV-1 integrase were studied by construction of an N-terminal fusion protein of the enzyme and GFP (GFP-IN) (Pluymers et al., 1999). Integrase is responsible for the integration of the retroviral cDNA into the cellular genome, and integration is an essential part of the retroviral replication cycle. However, the nuclear transport of integrase was not directly visualized before the construction of the fusion protein with GFP. Transient expression of GFP-IN in different mammalian cells allowed investigators to obtain solid evidence for the nuclear localization of the enzyme. Mutations in the conserved KRK motif (residues 186 to 189) that was suggested to be a putative nuclear localization signal did not affect the karyophilic property of HIV-1 integrase, as was shown by using GFP fusion protein (Tsurutani et al., 2000). Further mutational studies with the integrase-GFP construct may provide a better understanding of the nuclear transport mechanisms of integrase and of the whole preintegration complex.

Nef

Chimeric proteins consisting of HIV-1 Nef fused to a strongly fluorescing mutant of GFP were used to correlate Nef function with intracellular localization in human $CD4^+$ Jurkat T cells (Greenberg et al., 1997). The Nef-GFP fusion protein colocalizes with components of the clathrin coat, including clathrin and

the β-subunit of the AP-2 adaptor protein complex. The Nef-GFP protein was also found in the perinuclear region of the cell that was hypothesized to be the Golgi apparatus. Colocalization of Nef-GFP with components of the clathrin coat in the $CD4^-$ fibroblast cell line indicated that colocalization does not require expression of the CD4 molecule. Analysis of a panel of chimeric molecules containing mutant Nef moieties demonstrated that the N-terminal membrane targeting signal cooperates with additional element(s) in the disordered loops in the Nef molecule to colocalize the Nef protein with AP-2 adaptor complexes at the cell margin. This localization of Nef-GFP correlated with, but was not sufficient for, down-regulation of surface CD4. In T cells coexpressing CD4 and Nef-GFP, CD4 at the cell surface is redistributed into a discrete pattern that colocalized with that of Nef-GFP. This observation indicates that Nef may interact with a component of the AP-2-containing coat at this location. Welker and colleagues have shown that myristoylation and an N-terminal cluster of basic amino acids were required for virion incorporation and plasma membrane targeting of Nef (Welker et al., 1998). Fusion of the N-terminal domain of Nef alone to GFP led to membrane targeting and virion incorporation of the label protein, thus proving the high targeting potency of the sequence.

Human thioesterase II was demonstrated to bind to a site on HIV-1 Nef critical for CD4 down-regulation (Cohen et al., 2000). Both HIV-1 and SIV Nef-GFP fusion proteins were found to redistribute from cytoplasm and plasma membrane into peroxisomes in the presence of the thioesterase II, thus confirming high-affinity interaction between Nef and the enzyme.

A Nef-GFP fusion protein was used to follow microinjected cells in studies of the interaction of Nef with p21-activated kinase 1 (Fackler et al., 2000). The authors have used the fact that Nef fused to GFP retains full biological activity.

Gag proteins

The Gag proteins of HIV-1 are necessary and sufficient for the assembly of virus-like particles. The roles played by HIV-1 Gag proteins during the life cycle are complex and involve not only assembly but also virion maturation after particle release and early steps in virus replication. GFP fusion to the C-terminus of full-length or truncated Gag proteins was used for studying the interaction of the $Pr55^{Gag}$ molecule with the plasma membrane (Sandefur et al., 1998). This interaction is an essential step in the viral life cycle. The study was undertaken to identify a region outside of MA that determines efficient plasma membrane interaction of Gag polyprotein. Application of GFP tagging allowed simultaneous and rapid subcellular localization by fluorescence microscopy and quantification of proteins in subcellular fractions. The authors have used a human codon-optimized form of GFP and a vaccinia virus-T7 expression system because of it ability to readily produce Gag retrovirus-like particles. The study proved that membrane-targeting/binding function within MA by itself was insufficient to direct GFP to the plasma membrane. Extension of the Gag

region of the fusion protein to include CA and SP1 did not alter significantly the localization of the protein. When all or the N-terminal portion of nucleocapsid (NC) was included, fluorescence had punctate plasma membrane localization. The minimal region conferring plasma membrane localization (I domain) was identified as the N-terminal 14 amino acids of NC. Two arginine residues were found to be critical for the function of the I-domain (Sandefur et al., 2000). Thus, GFP labeling allowed straightforward identification of a domain involved in membrane targeting. An MA-GFP fusion protein was used to study the role of the HIV-1 viral protein U (Vpu) in the release of virus particles. Subcellular localization of Gag protein in the presence or absence of Vpu was studied with the use of subcellular fractionation techniques and by confocal microscopy with Gag-GFP fusion proteins (Deora et al., 2000). MA-GFP fusion protein showed enhanced membrane binding in the presence of Vpu, thus suggesting that Vpu action may be mediated by reinforcing Gag association with the plasma membrane. Gag fusion to GFP was also used in an attempt to understand the routing mechanism of the molecule from its site of synthesis in the cytoplasm to the plasma membrane (Perrin-Tricaud et al., 1999). Double labeling with actin-specific probe showed no colocalization of Gag with actin, suggesting that Gag may associate with other cytoskeletal components or that oligomerization of Gag occurs before it gets to the membrane.

GFP was also successfully used in the studies of cellular receptors involved in HIV-1 entry, mechanisms of their trafficking, interactions with viral envelopes, and impact of down-regulation on HIV-1 replication (Amara et al., 1997; Orsini, 1999; Tarasova et al., 1998). However, this topic was extensively reviewed recently (Kallal and Benovic, 2000; Pelchen-Matthews et al., 1999; Tarasova et al., 2000) and thus will not be detailed here.

CONCLUSION

GFP has already become an indispensable instrument in retroviral research. Many GFP-based tools have been developed and many of them are available for investigators through the AIDS Research and Reference Reagent Program established by the National Institute of Allergy and Infectious Diseases at the National Institute of Health. Because of their simplicity, GFP-based assays are becoming increasingly popular. It can be foreseen that application of GFP for studies of HIV will grow rapidly in the future. Many fluorescence-based methods of analysis continue to develop. More sensitive and selective methods of fluorescence detection and novel microscopic techniques, such as fluorescence correlation spectroscopy (Van Craenenbroeck and Engelborghs, 2000), fluorescence polarization spectroscopy (Fernandes, 1998), multiphoton laser scanning microscopy (Buehler et al., 1999), time-resolved fluorescence spectroscopy (Millar, 1996), and others stimulate even broader studies with application of GFP-tagged proteins and viruses. Fluorescent proteins with a variety of spectral properties (red, blue, or yellow proteins) with high quantum yields are

now available (Matz et al., 1999). They will allow wider application of GFP for fluorescence resonance energy transfer (FRET) for the studies of viral protein interaction with each other and with cellular proteins at different stages of viral life cycles. Novel instrumentation for high-throughput cell imaging and fluorescence detection will facilitate applications of fluorescent proteins in antiviral drug development.

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17

Flow Cytometric Analysis of Cells from Persons with HIV-1 Disease by Enzymatic Amplification Staining

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INTRODUCTION

Flow cytometry is a powerful analytical tool because it allows for efficient assessments of multiple characteristics on a large number of single cells in a reasonably short time. The capacity to analyze thousands of cells in less than a minute provides statistically significant information about cells in a heterogeneous population. Flow cytometry is a cell-by-cell analysis that provides information on individual cells in a diverse population so that structurally and functionally meaningful groupings can be visualized. These groupings can have profound significance for understanding biological events and consequently for making clinical decisions.

Flow cytometric analysis can assess the entire cell. Preparative techniques for detecting cell surface molecules, intracellular molecules, and specific nucleic acid sequences have been developed. Analysis of cell surface molecules is particularly interesting because the cells are intact and consequently the background is low. Moreover, the accumulation of molecules in the surface membrane is a concentrating mechanism that enhances the capacity for detection.

Nevertheless, the most important limitation of flow cytometric analysis of

surface molecules is the relative insensitivity of this technique compared with functional assays. The number of molecules required to be observed by flow cytometry has been estimated to be 2000–4000 molecules per cell (Loken and Herzenberg, 1975), but activity assays require only a few hundred molecules to be functionally active and significant. For a large number of important molecules, flow cytometric analysis has been uninformative.

ENZYMATIC AMPLIFICATION STAINING (EAS) FOR CELL-SURFACE MOLECULES

Cell-surface molecules can be detected with specific monoclonal antibodies directly conjugated with a fluorochrome. This technique, called direct staining, is easy to perform because it involves only one step: incubation with the labeled antibody and washing. Direct staining works well for molecules that are abundantly expressed on the cell surface and for which there are high-affinity antibodies available. Nevertheless, many cell-surface molecules cannot be adequately assessed by this technique.

Amplification of the fluorescent signal for the detection of cell-surface molecules by flow cytometry has been a long-sought-after goal. The major mechanism in current use for the amplification of these signals has been the application of multiple layers of receptor ligands with the final layer labeled with fluorochrome. This technique is generically known as indirect staining and represents an amplification over the staining obtained with fluorochrome-labeled primary antibodies.

The simplest scheme for amplification by layers is the use of a fluorochrome-labeled anti-immunoglobulin (Ig) binding to the Ig with specificity for the chosen cell-surface molecule. Amplification is achieved by multiple labeled anti-Ig binding to the bound primary antibodies, thereby increasing the number of fluorescent molecules fixed to the cell surface. A similar technique involves the use of biotinylated primary antibodies followed by avidin or streptavidin conjugated with the fluorochrome. Similarly, the amplification results from multiple, conjugated biotin molecules binding avidin or streptavidin conjugated with multiple molecules of fluorochrome.

Indirect staining has been further developed to include additional layers. Excellent results have been obtained with unconjugated primary antibodies followed by a layer of biotinylated anti-Ig antibodies and then by avidin or streptavidin conjugated to fluorochrome (Zola et al., 1990).

Another approach to the amplification of the fluorescent signal has been the development of new fluorochromes with brighter relative fluorescence intensities. Fluorescein isothiocyanate (FITC) was the first fluorochrome used widely for the analysis of cell surface molecules by flow cytometry. Fluorochromes with brighter relative fluorescence intensities include phycoerythrin (PE) and allophycocyanin. Peridinin chlorophyll protein is another commonly used

fluorochrome that is less bright than FITC. Conjugate fluorochromes, such as PE-cyanin 5 and PE-Texas Red among others, have been developed to enhance the fluorescent signal that can be obtained. The use of brighter fluorochromes has increased the capacity to distinguish positive and negative cells, although the effect is only in the range of a five-fold enhancement.

We have used a different approach to enhance the fluorescent signal in flow cytometry. Harnessing the potential of enzymes to amplify signals, we developed an enzymatically catalyzed reporter deposition system for flow cytometric analysis of cell-surface receptors (Kaplan and Smith, 1999). A similar system had been developed for immunoassays, immunohistochemistry, and in situ hybridization but had not been established for flow cytometry (Bobrow et al., 1989, 1991; van Gijlswijk et al., 1997). The system involves the conjugation of horseradish peroxidase (HRP) to the primary antibody. Alternatively, unconjugated primary antibodies or biotinylated primary antibodies can be used with HRP-conjugated anti-Ig or HRP-conjugated streptavidin, respectively. After HRP is localized on the surface of cells expressing the specific molecule of interest, peroxide is added with a labeled molecule, tyramide. In the presence of the enzyme with its substrate, it has been postulated that the phenolic compound, tyramide, forms a short-lived, highly reactive free radical that is able to react covalently with nearby electron-rich moieties such as tyrosines or tryptophans on the cell surface. Because the free radical does not persist long enough to diffuse away from the cell surface where it is formed, only the cell that has bound the enzyme is labeled with the tyramide. It should be noted that the labeled tyramide does not simply bind to the targeted molecule. Instead, it also binds to other molecules in the vicinity of this targeted molecule.

We have adapted this system of tyramide deposition for flow cytometry and have found that it can provide excellent enhancement of the fluorescent signal. CEM cells from a human T cell tumor line, were stained for surface expression of CD5 either by a standard amplification procedure (indirect staining) or by EAS. CD5 is a 67-kD surface glycoprotein of the scavenger receptor cysteine-rich family. It appears on T lymphocytes early in their development and is abundantly expressed on all mature T cells. It should be noted that the enzymatic amplification procedure differed from the standard procedure only by the inclusion of the enzyme-catalyzed reporter deposition steps. The primary antibodies and the fluorochromes were identical in both procedures. The results demonstrate that EAS gave a marked amplification of the fluorescent signal compared with standard amplification staining (indirect staining) (Fig. 17.1). The mean channel numbers for the cells stained with the specific anti-CD5 monoclonal antibodies were 7 for the standard amplification method and 751 for EAS. Cells stained with control Ig demonstrated mean channel numbers of 3 for the standard procedure and 4 for EAS. Thus, in terms of mean channel separation (specific antibody vs. isotype control antibody), EAS enhanced the fluorescent signal approximately 140 times over the standard technique. Similar experiments with cells from Molt-4 and HUT-102, other T-cell tumor lines

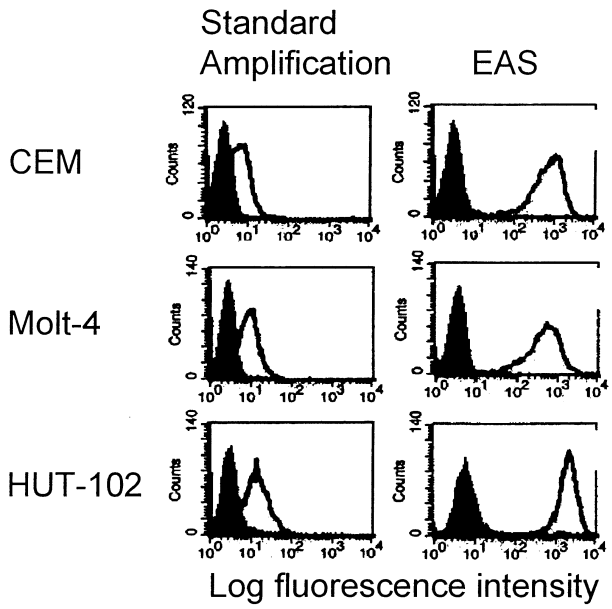

Figure 17.1. The human T-cell tumor lines CEM, Molt-4, and HUT-102, were stained with 5 ng of biotinylated control murine IgG1 (*closed histograms*) or 5 ng biotinylated anti-human CD5 (*open histograms*). The panels on the right represent cells stained with standard amplification procedures, and the panels on the left represent cells stained with EAS.

that express CD5, gave similar results. The channel separation between control and specific antibody peaks with standard amplification was 6 channels for Molt-4 cells and 12 channels for HUT-102 cells, whereas the channel separation with EAS (normalizing the control peak at the same channel number) was 409 channels for Molt-4 cells and 940 channels for HUT-102 cells. Excellent fluorescent signal enhancement has been obtained with every cell analyzed.

Moreover, we have tested antibodies to over 50 different molecules on various cells including tumor lines grown in culture, neoplastic lymphocytic cells ex vivo, peripheral blood mononuclear cells (PBMC) ex vivo, and PBMC activated and cultured in vitro. In every case, we have obtained marked amplification. The cell surface markers that we have analyzed include major histocompatibility complex (MHC) class I, MHC class II, CD3, CD4, CD5, CD6, CD7, CD25, CD34, CD45, CD79b, CD122, CD132, Ig kappa light chain, Ig lambda light chain, cytokine receptors, viral glycoproteins, and glycolipids, among others.

We have also performed comparative analyses with dilutions of the primary antibodies (Kaplan and Smith, 1999). These analyses suggested two important points to us. First, EAS allowed us to observe cell-surface molecules at antibody amounts that could not be used to detect these molecules using other

techniques of amplification. With certain levels of antibodies, we were able to separate histograms of specific vs. control antibodies with EAS that could not be distinguished by standard amplification procedures. Second, to obtain a desired level of channel separation, EAS required 10- to 100-fold less antibody than standard amplification techniques. With less antibody needed to obtain channel separation, we postulated that background staining would be minimized. Thus, we were encouraged to speculate that EAS could be used to detect molecules on the cell surface that had never been detected before.

The original impetus for our investigating enhanced signals in flow cytometry was the inability to detect surface Fas ligand by flow cytometry, although expression was easily detected in a bioassay (Kaplan and Smith, 1999). Testing a variety of transfected cell lines that did not show any specific staining for Fas ligand but that did possess Fas ligand activity, we were able to demonstrate Fas ligand surface expression by amplification staining (data not shown). To test our procedure in a rigorous way, we assessed PBMC that had been stimulated with phytohemagglutinin and interleukin (IL)-2 for 3 days of culture and then with phorbol myristic acetate (PMA) and ionomycin for 1 day. A similar protocol had been demonstrated to induce Fas ligand activity, but flow cytometric analysis of these cells did not demonstrate a definitive population of positive cells (Tanaka et al., 1996). Whereas we found that the standard staining technique did not demonstrate any expression of surface Fas ligand, amplification staining of these cells showed that 10–15% of the cells were expressing enough Fas ligand to define a separate positive subpopulation (Fig. 17.2*A*). It should be noted that the amplification method differed from the standard procedure only by the inclusion of the enzyme-catalyzed reporter deposition steps.

To ascertain that the staining we observed by the amplification procedure was significant in terms of the function of the cells, we sorted the positive population and the negative population and assessed their cytotoxic potential against Fas-expressing Jurkat targets (Sieg et al., 1996, 1997; Smith et al., 1998) (Fig. 17.2*B*). The results demonstrate that our amplification staining technique successfully identified functionally significant levels of Fas ligand on the cell surface. Moreover, they show that cells treated for amplification staining retained their viability and function, and further studies have demonstrated that the cells can continue to proliferate as well.

We have also used EAS to demonstrate annexin V staining of cells undergoing apoptosis. In this case, the staining was verified with an independent measure of apoptosis (Kaplan and Smith, 1999). Thus, with two different molecules whose presence could be verified independently by functional attributes, we have shown that EAS provides stainings that are significant. These data indicate that the histograms obtained by EAS are reliable and meaningful. Although in most cases it is difficult to validate staining because there is no readily measured independent parameter for most targeted molecules, verification of the staining of Fas ligand and phosphatidylserine gives us confidence in the application of EAS to other molecules.

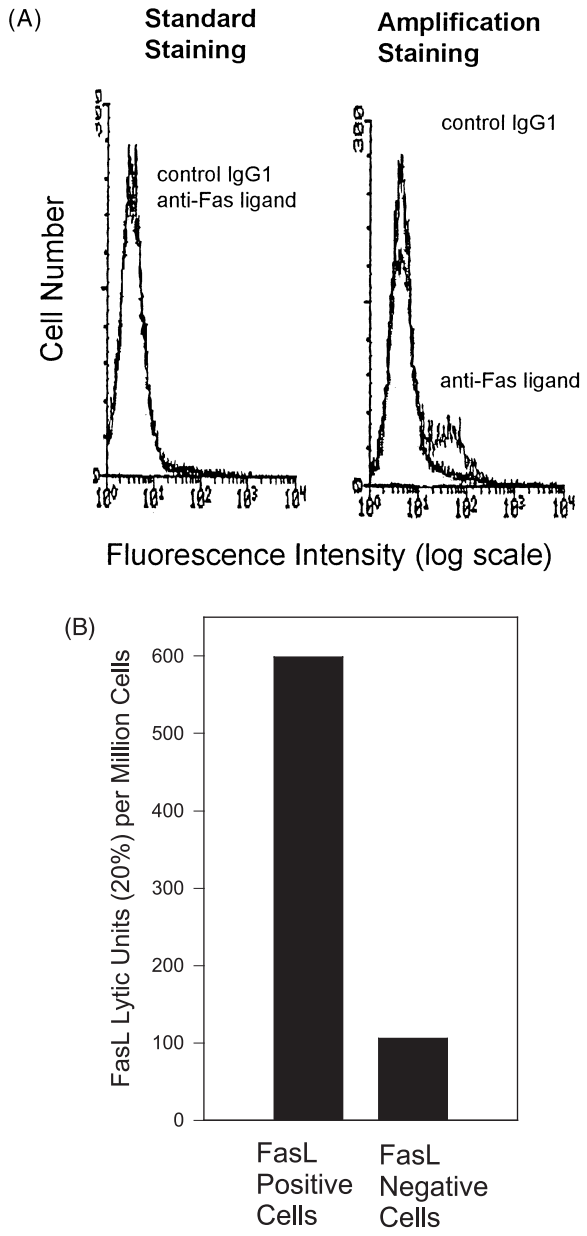

Figure 17.2. (**A**) Human PBMC induced to express Fas ligand were analyzed for surface expression of Fas ligand with biotinylated anti-Fas ligand Alf-2.1 monoclonal antibodies and control biotinylated murine IgG1 using either the standard technology (*left panel*) or enzymatic amplification technology (*right panel*). (**B**) The Fas ligand positive and negative cells were sorted on a EPICS Elite cell sorter (Beckman Coulter, Hialeah, FL). The sort was verified by reanalysis of the sorted cells. The Fas ligand positive and negative cells were assessed for Fas ligand apoptotic capability in a DNA fragmentation assay and the results shown in lytic units.

EAS FOR INTRACELLULAR MOLECULES

Intracellular molecules are also important to assess by flow cytometric analysis, but the difficulty with fluorescent signals for these antigens is even more pronounced than with surface molecules. Consequently, we have developed EAS for intracellular molecules. CEM cells were stained for bcl-2 using a specific murine monoclonal antibody. For the standard amplification procedure, we added an anti-murine Ig antibody conjugated with FITC. For EAS, we used a kit from Flow-Amp Systems, Ltd., (Cleveland, OH) which included the same fluorochrome for detection. Although the standard technique gave only marginal staining of bcl-2, we obtained excellent amplification of the fluorescent signal with EAS (Fig. 17.3). The mean fluorescence channel in the sample treated with an isotype control was 2 in the standard amplification method, whereas the mean fluorescence channel in the sample treated with the specific anti-bcl-2 monoclonal antibody was 3. In contrast, EAS gave mean fluorescence channels of 2 and 142 for the isotype control and the specific monoclonal antibody. Thus, EAS gave an approximate fold enhancement of approximately 140 compared with the standard amplification procedure.

We have succeeded in amplifying signals for organellar, cytoplasmic, and nuclear antigens. Similar to the situation with cell-surface molecules, amplification of the signal from more than 10 antigens has been achieved without encountering a single molecule whose signal could not be amplified.

USES OF EAS IN THE INVESTIGATION OF HIV-1 INFECTION

Flow cytometric analysis has played an important role in the investigation of infection by human immunodeficiency virus type I (HIV-1) from the beginning of the epidemic to the present time. In the initial characterization of the infection, flow cytometry was important in recognizing the selective loss of $CD4^+$ T lymphocytes from the peripheral blood. Analysis of the cell surface of monocytes revealed low-level CD4 expression and thereby explained the potential for these cells to be infected by the virus. Investigations of various induced cell surface molecules on $CD8^+$ T cells demonstrated a high level of expression of activation antigens, indicating the responsiveness of these cells to the virus. More recently, the availability of GFP-expressing recombinant HIV-1 has provided an important research tool for the identification of infected cells. Moreover, the construction of tetramers of MHC class I molecules and HIV-1 peptides has demonstrated the large proportion of $CD8^+$ T cells from the peripheral blood of infected persons that have specificity for the virus.

Nevertheless, there are many circumstances in the investigation of HIV-1 disease with flow cytometric analysis that may be clarified with the enhanced resolution of EAS.

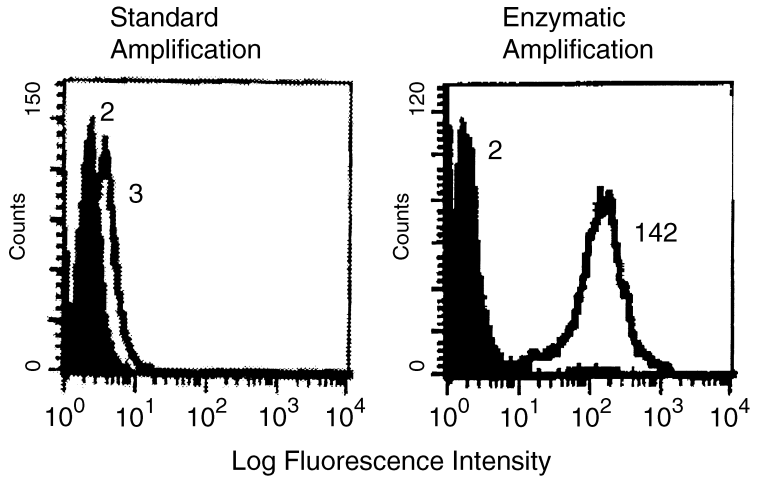

Figure 17.3. CEM cells were fixed and permeabilized and then stained with 100 ng of murine IgG1, an isotype and subtype control (*closed histograms*) or with 100 ng of murine anti-bcl-2 monoclonal antibody (*open histograms*). The stained cells were processed by standard amplification techniques (*left panel*) or by EAS (*right panel*). The mean channel numbers of the histograms are shown.

Recognition of Viral Gene Products

HIV-1 produces many different proteins: gp120 Env, gp41 Env, p17 Gag, p9 Gag, p24 Gag, p11 Pol (protease), p66/p51 Pol (reverse transcriptase), p31 Pol (integrase), Nef, Vif, Vpr, Vpu, Tat, and Rev. Moreover, these proteins are produced and expressed in infected cells according to a viral program that relates to the life cycle of the virus and consequently to the pathogenesis of the infection. Many of these proteins are regulatory and are not produced in large amounts. The expression of structural proteins is also interesting because their production signifies viral production. It is important to assess low-level viral production, particularly in the setting of antiviral therapies. Also, gp120 is a membrane protein expressed on the cell surface and thus it represents a tag for an infected cell. It is likely that the enhanced fluorescent signal that can be obtained with EAS for both cell surface molecules and intracellular molecules can be used to assess viral protein production in HIV-1 infected cells.

In several studies, investigators have suggested the possibility that HIV-1 can exist as a latent infection (Borvak et al., 1995; Chun et al., 1997; Emiliano et al., 1996; Finzi et al., 1999). Latency at a cellular level has been proposed to account for an organismal phenomenon: the recrudescence of viral particles in the serum after successful antiviral therapy. But the possibility of latency of the virus in the organism has not been translated in terms of a latent cellular infection such as exists for herpes virus infections. Does HIV-1 exist as a latent cellular infection? If so, which viral proteins are produced in this form of

latency? Several cell lines such as ACH-2, J1.1, U-1, and OM-10.1 have been proposed to represent cells latently infected with HIV-1 (Butera et al., 1991, 1994; Clouse, et al., 1989; Perez et al., 1991). These cells produce large amounts of virus after exposure to inducing agents such as cytokines or phorbol esters. Do they produce a different set of viral proteins before activation than after activation? This question is crucial because it may provide insights on the mechanisms that the virus uses to survive during antiviral therapy and most importantly because it may provide targets for eliminating the persistent infected cells. In a population of cells, are there cells that are latently infected and other cells that produce low levels of virus? A single-cell analysis of the population is required to answer this question.

Flow cytometric analysis of HIV-1 proteins with standard amplification technologies has not been informative. For instance, most investigators who have assessed antibodies to gp120 have not used flow cytometric analysis but have instead relied on neutralization assays and enzyme-linked immunosorbent assay (ELISA). One of the most important difficulties with flow cytometric analysis for HIV-1 gp120 has been availability of antibodies with sufficient reactivity to bind to the various isolates of the virus. In one sense, this problem is also a reflection of the poor sensitivity of flow cytometry inasmuch as a more sensitive technology would be able to detect antibodies that bind with lower affinities.

One of the first antibodies used for the assessment of the expression of viral proteins by flow cytometry was the human monoclonal antibody F105 that is specific for the CD4 binding site of gp120 (Posner et al., 1991). F105 is a human IgG1 κ monoclonal antibody that was obtained by using Epstein-Barr virus to transform B cells derived from an HIV-1 infected person. This antibody has been used to assess gp120 expression by flow cytometry. gp120 from all strains tested, including MN, RF, IIIB, and SF2, were bound by this antibody. Nevertheless, the histograms obtained were not impressive; it is not even clear that the antibody could distinguish infected from noninfected cells, an important criterion for usefulness. The use of F105 to analyze gp120 expression has not been proven to be useful.

Other human monoclonal antibodies specific for HIV-1 gp120 have been used in flow cytometric analysis and have given excellent histograms with cell lines stably infected with HIV-1 (Alsmadi et al., 1997). Uninfected cells or infected cells without primary antibody were used as controls. Unfortunately, an isotype/subtype control was not included in the analysis. These antibodies were shown by flow cytometry to recognize gp120 from native primary isolates from clades B and E as well as various strains such as SF2 and RF. Nevertheless, gp120 from strain MN was not recognized. These antibodies seem promising; however, no additional publications with them have been listed in PubMed since 1997.

Another study with human monoclonal antibodies specific for HIV-1 gp120 demonstrated the possibility that these antibodies could be used in flow cytometric analyses to serotype primary isolates (Zolla-Pazner et al., 1995). Two of

the antibodies tested gave excellent levels of fluorescence intensity, and three other antibodies gave marginal shifts. However, the significance of these findings is not clear because no isotype/subtype controls were shown. Moreover, PubMed lists no additional investigations with these antibodies, although a similar antibody has since been described that binds to clade E isolates and cross-reacts with viruses from other clades (Gorny et al., 1998).

Other investigators have obtained murine monoclonal antibodies specific for HIV-1 gp120 that have been used for flow cytometric analysis (Laman et al., 1992; Moore et al., 1993; Pincus et al., 1989). A murine monoclonal antibody specific for HIV-1 gp120 of strain IIIB was obtained and shown to produce histograms that were clearly shifted in comparison to both isotype control-stained infected cells and specific antibody-stained uninfected cells (Pincus et al., 1989). Another murine monoclonal antibody was obtained and tested by flow cytometry (Laman et al., 1992). This antibody, IIIB-V3-13 produced a shift in fluorescence comparing infected with uninfected cells, but no isotype/subtype control was included. Moreover, the shift obtained, seen with fluorescence read on a linear scale, was marginal at best. Eight different murine monoclonal antibodies were used to probe the available structure of oligomeric gp120 on the cell surface of H9 cells transfected with a HIV-1 molecular clone (Moore et al., 1993). Isotype control immunoglobulins were not used in this study. Unfortunately, no histograms were shown.

Analysis of intracellular viral antigens has not been any more successful. Most investigators have assessed the structural protein, HIV-1 p24, in PBMC from infected patients (Costigliola et al., 1992; Holzer et al., 1993; McSharry et al., 1990; Ohlsson-Wilhelm et al., 1990). These studies were conducted to obtain a relevant marker of disease progression in the patients, and high proportions of p24-expressing $CD4^+$ cells (up to 25%) were found in infected patients compared with the levels of infected cells detected by sensitive polymerase chain reaction (PCR) techniques. The failure to obtain independent confirmation of expression was worrisome. In fact, cells found to be expressing p24 by a flow cytometric technique were found to be no more likely to contain HIV-1 provirus than cells determined to be negative for p24 expression by flow cytometry (Kux et al., 1996). The high levels of p24 expression in $CD4^+$ cells may have been the result of the failure of the investigators to include isotype/subtype controls in their studies along with the relatively high levels of non-antigen-specific binding found in cells from HIV-1-infected persons compared with healthy volunteers. Moreover, in this same study it was clear that patient cells did not include definitive subsets expressing p24, which would produce a bimodal distribution of fluorescence intensities. Rather there were shifts in the entire population of cells (Cameron et al., 1998). It is easy for proportions of positive cells to be fallacious in the situation where entire populations shift and a somewhat arbitrary cut-off value is used to assign positivity.

Flow cytometry, for either cell surface viral glycoproteins or intracellular viral proteins, has not been found to be useful in studies of the expression of HIV-1 viral proteins. Nevertheless, the availability of this technology would

enhance our capabilities to analyze HIV-1 disease. The identification of cells that express viral proteins, the determination of the set of viral proteins expressed at a given time, and the analysis of variant viral strains are important and significant goals for investigators to achieve.

It is probably correct to assume that flow cytometric analysis of HIV-1 proteins by flow cytometry has not been successful because the available antibodies are not sufficient to detect viral proteins given the current sensitivity of the technology. EAS may be helpful because it offers a significant enhancement in the resolving power of flow cytometry. Moreover, EAS may also be used to find new antibodies that would be informative but that have been discarded because they did not work with the standard procedures for analyzing the cells.

We have used human anti-HIV-1 gp120 monoclonal antibodies to stain the "latently" infected cell line ACH-2. The cells were processed either by a standard amplification procedure or by EAS (Fig. 17.4). The results demonstrate that EAS allows for the detection of heterogeneous levels of gp120 on the surface of most ACH-2 cells. The uninfected parental cell line showed no specific staining. The standard staining procedure did not detect most of the gp120-expressing cells. It should be noted that another control was included: an anti-Ebola virus antibody gave histograms identical to the control IgG1 (data not shown). Also, similar results were obtained with another "latently" infected cell line, J1.1, and its parent, Jurkat (data not shown). Finally, using EAS we also

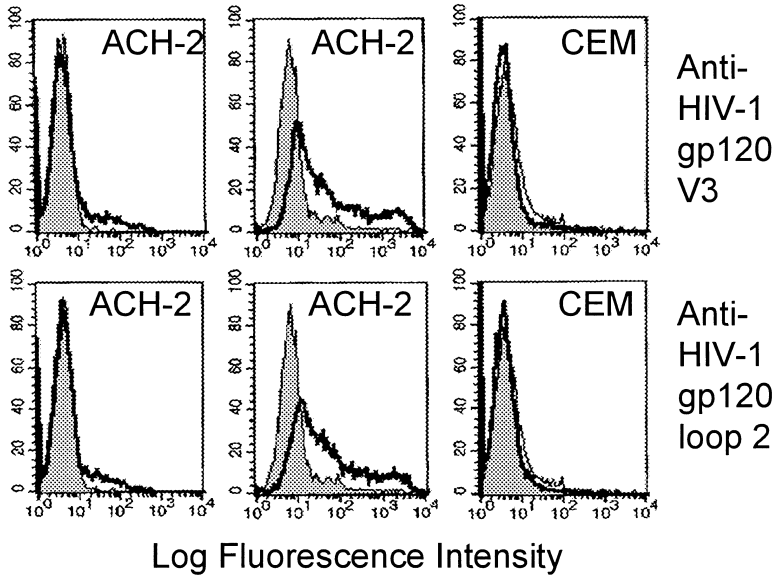

Figure 17.4. ACH-2, "latently" infected with HIV-1, and CEM, the uninfected parental line to ACH-2, were stained with two different anti-gp120 human monoclonal antibodies that had been biotinylated (*open histograms*) or with control biotinylated IgG1 (*filled histograms*). The cells were processed for flow cytometric analysis by standard amplification (indirect staining; left panels) or by EAS (middle and right panels).

detected intracellular p24 in both ACH2 and J1.1 cells (data not shown). With EAS technology, the question of viral latency might be more definitively addressed.

Recognition of Chemokine/Cytokines and Their Receptors

Chemokines and cytokines and their receptors are important players in HIV-1 disease pathogenesis. Most importantly, chemokine receptors serve as coreceptors with CD4 for HIV-1 infection of cells. The significance of chemokine receptors is validated by the relative resistance of persons with a 32-bp deletion in the gene encoding the CCR5 receptor (Sampson et al., 1996). Moreover, a valine to isoleucine substitution at position 64 in the CCR2 receptor has been associated with a significant delay in HIV-1 disease progression (Kostrikis et al., 1998).

Production of chemokines has also been shown to influence HIV-1 disease pathogenesis. Again, genetic analysis has provided important results that help to substantiate the role of chemokines. A common genetic polymorphism of stromal-derived factor-1, which is the principal ligand for CXCR4, an important HIV-1 coreceptor, was found to be associated with delayed disease progression (Winkler et al., 1998). Furthermore, β-chemokines inhibit the replication of HIV-1 in vitro (Arenzana-Seisdedos et al., 1996; Bleul et al., 1996; Oberlin et al., 1996; Schols et al., 1997). These findings are likely to be significant because spontaneous and antigen-induced production of HIV-inhibitory β-chemokines by PBMC from HIV-1-infected persons has been found to be associated with better clinical status (Garzino-Demo et al., 1999; Saha et al., 1998).

The roles of chemokines and their receptors in the pathogenesis of HIV-1 disease indicate the importance of analyzing these molecules; however, both the chemokines and their receptors are difficult to assess. Chemokines are highly active molecules and are produced and secreted in small quantities. There are few reports of successful analysis of chemokines using flow cytometric analysis for intracellular antigens. A search of the PubMed database revealed only one such reference, which involved the analysis of IL-8 in human mast cells (Grutzkau et al., 1997). Nevertheless, these investigators were not stimulating the production of the chemokine; mast cells store these substances in secretory vesicles. For lymphocytes and monocytes that are stimulated to produce chemokines, the difficulty in assessing production using flow cytometry for intracellular antigens may be significant. The absence of publications describing this technology suggests that it has been difficult to obtain high-quality data with the available protocols. It is likely that the difficulty in assessing cells for the production of chemokines involves the poor sensitivity of the standard procedures of flow cytometric analysis. Consequently, EAS may enable investigators interested in HIV-1 disease pathogenesis to assess chemokine production in lymphocytes and monocytes.

Likewise, chemokine receptors are expressed and are active in low copy

number per cell. Often, investigators analyzing cells for chemokine receptors obtain histograms that are not clear and definitive. The histograms that represent cells stained with the isotype and subtype control Ig and histograms that represent cells stained with the antibodies specific for chemokine receptors are not clearly distinct from each other. There may be shoulders or smears or shifts that make the interpretations of these data difficult and tentative. In other cases, a failure to demonstrate any difference between the histograms obtained with control and specific Ig is dubious. The sensitivity of flow cytometry for the surface expression of molecules has been measured to be approximately 2000 molecules per cell (Loken and Herzenberg, 1975), but signal transduction through receptors has been shown to be more in the order of several hundred molecules per cell (Harding and Unanue, 1990). Thus, the failure to detect expression using a suboptimal technology does not mean that the targeted molecule, such as chemokine receptors, is not present or that it is not active.

Again, EAS has the potential to clarify the data concerning chemokine receptor expression. The added resolving power of EAS can be used to definitively assess the expression of chemokine receptors on specific cells. Clear-cut, distinct peaks can be obtained with EAS and these data, which are easier to interpret and thereby have the potential to give us a more accurate description of the expression of these important molecules. It is likely that some of the difficulties that have been encountered in detecting chemokine receptors on cells have been related to antibodies that do not possess affinities sufficient to support the protocol. EAS may render these low-affinity antibodies more useful and may thereby widen the reagents that can be utilized in the analysis of cell surface expression of chemokine receptors.

Cytokines and their receptors have also been shown to be important in HIV-1 pathogenesis. Several cytokines, tumor necrosis factor (TNF)-α (Israel et al., 1989), IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (Koyanagi et al., 1988), are known to enhance viral production. Other cytokines induced by infection, such as IL-10 (Akridge et al., 1994; Borghi et al., 1995), IL-16 (Zhou et al., 1997), IL-13, and interferon (IFN)-γ (Bailer et al., 1999), have been shown to inhibit viral replication. The expression of many of these cytokines that influence viral production are also induced by viral proteins. Moreover, many cytokines have been implicated in the immune response to the virus (Appay et al., 2000). The production of cytokines by antigen-specific cells has also been used to track these cells (Pitcher et al., 1999). Thus, the detection of cytokine-producing cells and cytokine receptor-expressing cells is valuable in the investigation of HIV-1 disease pathogenesis.

Like chemokines, cytokines and their receptors are active at low copy numbers per cell. Consequently, they are often difficult to assess accurately and sensitively on a cell-by-cell basis. For these reasons, it is likely that analyses of cytokines and cytokine receptors by flow cytometry would benefit from the greater sensitivity and resolving power that has been demonstrated by EAS.

Recognition of Lymphocytic Markers

HIV-1 disease pathogenesis has been characterized by marked alterations in the physiology of lymphocytic cells. These alterations are either a consequence of the massive immune responses that are induced by the chronic viral infection or direct effects of viral products on cellular proteins. The continuous presence of virus constitutively stimulates the cells of the immune system and thereby results in phenotypic changes in the cells. For instance, $CD8^+$ T lymphocytes express enhanced cell-surface levels of the T-cell activation markers CD38 and CD95. Conversely, it has been reported that circulating $CD8^+$ T cells have decreased levels of CD3ζ and CD28 (Trimble et al., 2000). Intracellular molecules also fluctuate during HIV-1 infection. For instance, levels of bcl-2 and thioredoxin in monocytes are decreased in HIV-1 seropositive individuals with $CD4^+$ T cell counts above 500 μl/microliter. However, in patients with low levels of $CD4^+$ T cells, monocytic bcl-2 is expressed in amounts commensurate with monocytes from uninfected persons and thioredoxin levels in these cells are elevated (Elbim et al., 1999).

Besides the widespread consequence of the presence of large amounts of foreign molecules, cellular infection with the virus also has marked, direct effects on host protein expression. For instance, HIV-1 Tat has been shown to increase metalloproteinase production (Lafrenie et al., 1996), to increase CXC-chemokine receptor 4 expression (Secchiero et al., 1999), to induce CD69 surface membrane display (Blazquez et al., 1999), to enhance the level of a hematopoietic cell-specific transcription factor and down-regulate the chemokine MIP-1α (Sharma et al., 1996), to increase Bcl-2 expression (Wang et al., 1999), and to up-regulate IL-4 receptors (Husain et al., 1996). Both HIV-1 Nef and Vpu inhibit the surface expression of CD4 and MHC class I molecules (Aiken et al., 1994; Collins et al., 1998; Kerkau et al., 1997; Schubert et al., 1998; Schwartz et al., 1996). Nef also inhibits CD25 and c-myb (Greenway et al., 1995). HIV-1 Vpr acts to induce I-κB and Bcl-2 while inhibiting the expression of Bax (Ayyavoo et al., 1997; Conti et al., 1998). Besides these definitive changes in the expression of cellular proteins attributable to specific viral proteins, there are many other changes that have been demonstrated that have not been clearly assigned to specific viral gene product. HIV-1 infection causes significant, widespread changes in the expression of cellular proteins, and these changes are important in HIV-1 disease pathogenesis.

The analysis of phenotypic changes in lymphocytes and other blood cells from patients with HIV-1 disease have been necessarily limited to the detection levels of the available technologies. Flow cytometric analysis is particularly useful for analyzing these changes because it provides a cell-by-cell assessment. Because these changes are often seen in subsets of the total cells, a cell-by-cell analysis, which can also provide a more extended phenotypic analysis, is desirable. EAS is able to provide flow cytometry with a considerably greater sensitivity and thereby greater resolving power. Consequently, enzymatic amplifica-

tion is likely to be useful for assessing phenotypic changes that occur in cells from infected persons.

CONCLUSION

The investigation of HIV-1 infection and disease pathogenesis has been significantly affected by the capacity to analyze single cells by flow cytometry. The technology provides for fast and efficient analysis of large numbers of cells, measuring for multiple parameters simultaneously. Moreover, the capability of sorting selected populations based on the analysis on a flow cytometer adds a significant dimension to the potential for investigating the consequences of HIV-1 infection in vivo and in vitro.

Nevertheless, flow cytometric analysis has been hampered by a major limitation: the poor sensitivity of the analysis compared with other techniques. Most importantly, many molecules are expressed in functionally significant amounts that cannot be detected by the most sensitive current flow cytometric methods.

Enzymatic amplification technology allows for a 100-fold enhancement of the fluorescent signal and, consequently, the expression of molecules that could not be detected by standard procedures can now be observed. To be able to track the expression levels of functionally significant molecules on a cell-by-cell basis in an efficient and timely manner represents a major improvement in the technical capabilities for investigators working to understand the pathogenesis of HIV-1 disease.

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Antigen-Specific Cytokine Responses in HIV Disease

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CYTOKINES AS MARKERS FOR T-CELL FUNCTION IN HIV DISEASE

A number of viral pathogens including human immunodeficiency virus (HIV) have the capacity to establish chronic, often permanent, infection in which effective host immunity serves to contain the pathogen and prevent disease, but does not eliminate the infection. Many recent studies have provided evidence for the role of both $CD4^+$ and $CD8^+$ effector T cells in maintaining immunity in chronic viral infections. However, the critical contribution of the antigen-specific responses within these major subpopulations remain unclear (Altfeld and Rosenberg, 2000; McMichael, 2000; Picker and Maino, 2000). HIV-1 specific T-cell responses have been extensively investigated in untreated, chronically HIV-1-infected subjects using traditional proliferation or long-term cytotoxic T lymphocyte (CTL) assays. In general, such responses have been either undetectable or low (stimulation indices ≤ 5) in the vast majority of subjects, regardless of the stage of disease (Musey et al., 1999; Rosenberg and Walker, 1998; Valentine et al., 1998). These responses were largely directed at gag, although responses directed towards env and pol determinants could sometimes be detected (Haas et al., 1998; McAdam et al., 1998; Picker and Maino, 2000; Rosenberg et al., 1997; Rowland-Jones et al., 1998).

In the analysis of these studies, it is important to note that proliferation and

CTL assays do not provide quantitative information on the frequency of antigen-specific T cells. The readout of such assays reflects both the absolute number of responsive cells plated in the assay and the ability of these cells to exponentially expand over the 5–6 days of in vitro incubation. The latter may not be constant from sample to sample or assay to assay, particularly in the setting of HIV infection, where active disease may be associated with 1) viral infection and consequent destruction of the cells responding in the assay; 2) an immune hyperactivation state that predisposes T cells to apoptosis, particularly in vitro; and 3) the presence of viral products with inhibitory/apoptosis-inducing activity (Cohen et al., 1999; Shearer, 1998). In addition, long-term assays may underestimate antigen-specific frequencies when the percentage of $CD4^+$ cells in the mononuclear preparations is low, decreasing the absolute number of responding cells in each analysis.

Recently, more quantitative functional assays have been developed that have distinct advantages over the traditional proliferation and cytotoxicity assays. Among these are cytokine flow cytometry (CFC) (Pitcher et al., 1999; Suni et al., 1998; Waldrop et al., 1997) and enzyme-linked immunospot (ELISPOT) assays (Czerkinsky et al., 1983; Hutchings et al., 1989) for detection of individual cytokine-secreting cells. Immunofluorescent staining with major histocompatibility complex (MHC)-peptide molecular complexes (''tetramers'') has also been used as a simple and rapid method for assessing the presence of T cells specific for single epitopes of pathogenic importance (Altman et al., 1996; Gray et al., 1999; Allen et al., 2000; Gray et al., 2000; Allen et al., 2001). However, it is not a functional test, as it reveals only the specificity of a population of T cells as determined by their ability to recognize a particular peptide bound to a particular MHC molecule. This limits the clinical utility of tetramers as monitoring tools, because they are restricted by epitope and by MHC allele, and because specificity is not always correlated with function in certain disease states (Goulder et al., 2000).

Both $CD4^+$ and $CD8^+$ memory T cells respond to appropriate antigen stimulation with the rapid production of a number of effector and/or immunoregulatory cytokines involved in maintaining antiviral immunity. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α expression, for example, represent canonical activation markers for identifying antigen-specific T cells (Maino et al., 2000; McMichael et al., 2000; Picker and Maino, 2000; Pitcher et al., 1999). To this end, we have taken advantage of the fact that both $CD4^+$ and $CD8^+$ memory T cells respond to appropriate antigen stimulation with the rapid production of effector and/or immunoregulatory cytokines to develop a flow cytometric approach for the qualitative and quantitative assessment of antigen-specific cells. Flow cytometric detection of cytokine expression (CFC) by CD4 and CD8 T lymphocytes in response to specific antigen not only serves as a marker for quantitative assessment of cell-mediated immunity but also enables a qualitative assessment of cytokine expression by individual cells. More recently, this approach has been adapted to allow determination of 1) complete pathogen $CD4^+$ and $CD8^+$ T-cell responses in clinical samples (Maino and

Picker, 1998; Picker and Maino, 2000; Sester et al., 2000); 2) the array of specific class I and II epitopes to which the individual responds (Kern et al., 1998, 1999; Maecker et al., 2001) and 3) the number and relative hierarchy of T-cell receptor (TCR)-defined clonotypes involved in these pathogen-specific responses (Bitmansour et al., 2001).

CD4 T-CELL CYTOKINE RESPONSES TO HIV ANTIGENS

Although viral-specific $CD4^+$ T cells have proven to be a critical component of the immunologic control of chronic viral infections in a number of infectious disease models (Asanuma et al., 2000; Li et al., 1998; Pitcher et al., 1999; Rosenberg and Walker, 1998; Rosenberg et al., 1997; Waldrop et al., 1997), the role, and even the existence of, HIV-1-specific $CD4^+$ T cells in human HIV-1 infection has been controversial. Recently, a number of studies have provided new evidence for a significant role of $CD4^+$ T cells in maintaining HIV immunity. Pitcher and colleagues (Pitcher et al., 1999) demonstrated that significant frequencies of $CD4^+$ T cells expressed IFN-γ and TNF-α in response to HIV antigens in the majority of subjects with active HIV-1 infection. It was further demonstrated that overall frequencies of functional, HIV-1-specific $CD4^+$ memory T cells were considerably diminished in a cohort of long-term highly active antiretroviral therapy (HAART)-treated subjects with chronic HIV infection, suggesting that prolonged viral suppression is associated with a decline in HIV-1-specific $CD4^+$ T-cell memory. These observations were subsequently confirmed in a separate study (Maino et al., 2000) using similar methodology that further demonstrated that vaccinations of such HAART-treated subjects with a gp-120-depleted HIV-1 immunogen rapidly enhanced frequencies of HIV-specific $CD4^+$ T cells expressing either TNF-α or IFN-γ, either by expansion of the remaining HIV-1-specific memory cells or by recruitment of new HIV-1-specific memory cells. The inability to develop HIV-specific $CD4^+$ T-cell cytokine responses in chronic HIV infection despite effective suppression of viral replication may suggest that memory T cells specific to the virus are preferentially lost very early after seroconversion.

Alternatively, recent studies examining similar methodologies for measurement of T helper immune responses suggest these data are consistent with the model that the memory T-cell population is in constant flux, with the size of any given antigen-specific T-cell clonotype being continuously influenced by the local antigen concentrations in the tissues and, most significantly, by competition with other clonotypes (Andersson et al., 1998; Cavert et al., 1997; Haase, 1999). Other reports have suggested that the immune repertoire against the virus may decrease in response to potent antiviral drug therapy (as the antigenic stimulus is removed) but memory HIV-specific immune function remains a measurable parameter (Pitcher et al., 1999). Such findings are consistent with the concept that low-level HIV-specific memory T cells persist in chronic infection. A major implication of these findings is that in the absence of HAART

"rescue" during acute infection, the majority of HIV-1$^+$ subjects (i.e., those destined to become progressors) exhibit very low to undetectable frequencies of HIV-1-specific CD4$^+$ T cells (determined by the intracellular cytokine assay) very early in the course of infection, presumably due to the developing CD4$^+$ memory/effector T cells being killed by direct HIV-1 infection and/or HIV-1-associated apoptosis.

CD4 T-CELL CYTOKINE RESPONSES TO CMV IN HIV DISEASE

Cytomegalovirus (CMV) infections are often associated with opportunisitic infection in HIV disease, especially in untreated individuals. Studies by Waldrop and co-workers (Waldrop et al., 1997) demonstrated that HIV-seropositive subjects averaged over threefold higher frequencies of polarized, predominately IFN-γ- and TNF-α-producing, CMV-specific CD4$^+$ T cells. Significantly, these seropositive subjects demonstrated diminished effector frequencies for heterologous, nonubiquitous viruses such as mumps. Taken together, these data suggested the existence of homeostatic mechanisms capable of selectively preserving memory T-cell populations reactive with ubiquitous, opportunistic pathogens such as CMV at the expense of memory populations reactive with more sporadically encountered infectious agents. An important conclusion of this study was that, in HIV disease, protective immunity to particularly common pathogens may be maintained in a setting of decreasing overall CD4$^+$ T cells by increasing the relative clonal representation of T cells reactive with such pathogens (Mittler et al., 1996; Selin et al., 1996).

Komanduri and colleagues (Komanduri et al., 1998, 2001) used similar intracellular cytokine detection methodology for enumeration of antigen-specific memory T cells in a later study and provided evidence that the presence of active CMV-associated end organ disease (EOD) strongly correlated with loss of CMV-specific CD4$^+$ T-cell responses. Patients with no history of EOD or patients with quiescent EOD following HAART demonstrated strong CMV cytokine responses. These studies highlight the significance of this assay for the understanding of the homeostatic mechanisms involved in disease and suggest additional applications for monitoring immune reconstitution in other clinical conditions where the immune system is compromised.

CD8$^+$ T-CELL CYTOKINE RESPONSES TO HIV ANTIGENS

In response to infection, CD8$^+$ T cells expand and differentiate into effector cells, which mediate pathogen clearance through perforin-dependent cytolytic activity or expression of IFN-γ or TNF-α (Harty et al., 2000). CD8$^+$ T-cell responses have been strongly implicated in the maintenance of immunity to HIV. In normal immune responses, following pathogen clearance, the majority of antigen-specific CD8$^+$ T cells undergo cell death (apoptosis) in which 80–90% of the expanded population are eliminated, irrespective of the magnitude

of the intitial expansion. The remaining antigen-specific memory CD8$^+$ T cells are maintained throughout the life of the host.

In HIV disease, HIV-specific CD8$^+$ T cells play a major role in the control of virus during HIV primary infection but in most infected individuals do not completely prevent viral replication. The recent introduction of more quantitative methods for measuring antigen-specific CD8$^+$ T cells, including the use of MHC-peptide complexes and cytokine flow cytometry, has enabled a more precise analysis of the CD8$^+$ T-cell response in HIV disease. A number of recent studies have examined CD8$^+$ T-cell cytokine responses to specific peptides or peptide mixtures (Appay et al., 2000; Betts et al., 2000; Dalod et al., 1999; Goulder et al., 2000; Kern et al., 1998) to define specific epitopes targeted by CTL (Dalod et al., 1999; Donahoe et al., 2000; Kern et al., 1998; Maecker et al., 2001) and to characterize CD8$^+$ T-cell repertoires in HIV disease (Dalod et al., 1999; Goulder et al., 2000; McMichael et al., 2000; Sousa et al., 1999). These studies have taken advantage of the multiparameter capability of flow cytometry for simultaneous quantification of HIV-specific CD4$^+$ and CD8$^+$ T cells. Figure 18.1 illustrates four-color flow cytometric analysis of both CD4$^+$ and CD8$^+$ T-cell responses to mixtures of 15-mer overlapping peptides derived from the sequences of p55 Gag protein of HIV or pp65 protein from human cytomegalovirus (HCMV). This analysis allows the comparison of the relative contribution of CD8$^+$ and CD4$^+$ T cells to the maintenance of virus-specific T cell immunity. In asymptomatic HIV seropositive individuals, for example, high frequencies (5–20%) of HIV antigen-specific CD8$^+$ T cells were observed when compared with the lower frequencies (0.1–3%) of specific CD4$^+$ T-cell responses. The CD8$^+$ T-cell responses, however, were more variable over time compared with the more stable CD4$^+$ T-cell responses (unpublished observations).

By using overlapping peptides spanning an entire protein sequence, CD8$^+$ T-cell responses can be detected to multiple epitopes, regardless of human leukocyte antigen (HLA) type. Although 9 amino acid peptides produce the strongest CD8 responses, 15 amino acid peptides can produce similar levels of CD8 responses, while also generating CD4 responses. An algorithm developed by Florian Kern (Kern et al., 1998), in which consecutive 15-mer peptides overlapping by 11 amino acids, has been used to effectively detect epitopes recognized by both CD4$^+$ and CD8$^+$ T cells and to detect T-cell responses to specific viral proteins (Kern et al., 1998; Maecker et al., 2001; Picker and Maino, 2000). Figure 18.2 shows a typical whole blood cytometric analysis of a CD8$^+$ T-cell response against peptide mixtures derived from the major HIV proteins from an HIV-seropositive individual.

Using this approach, investigators can assess a number of important features of CD8$^+$ effector/memory T-cell responses to HIV. Thus, not only can the magnitude of the total CD8$^+$ T-cell response be quantitated, but, in addition, comparison of responses to individual subunits and epitopes can be analyzed. Goulder and colleagues (Goulder et al., 2001b) recently demonstrated, using CFC in longitudinal studies, that CTL responses that are present in chronic HIV infection may differ substantially from those that constitute the initial antiviral responses. Appay and co-workers (Appay et al., 2000) combined the

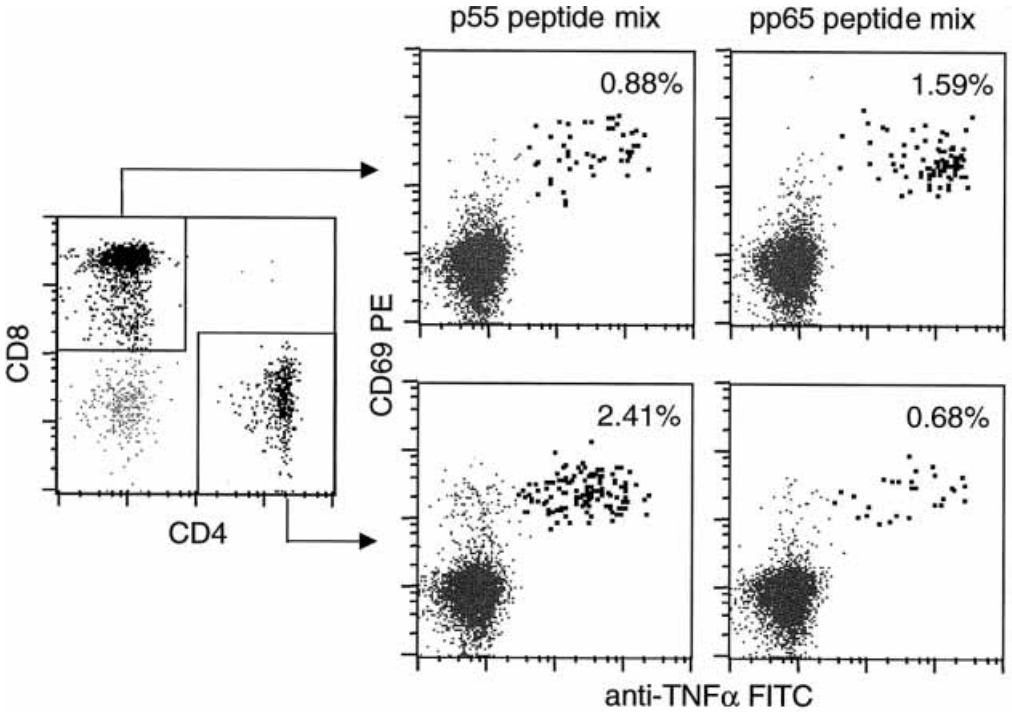

Figure 18.1. Simultaneous detection of $CD4^+$ and $CD8^+$ T-cell cytokine responses to peptide mixtures derived from sequences of p55 gag and pp65 protein subunits of HIV and HCMV. Whole blood from an HIV and CMV seropositive donor was stimulated for 6 h using 15-mer overlapping peptide mixtures shown at the top. CD8-gated (*top row*) and CD4-gated (*bottom row*) responses are shown. Note that in this example the pp65 15-mer peptide mix stimulates a significantly higher CD8 response than was observed for the $CD4^+$ T-cell response. In contrast, the HIV response was predominantly observed within the $CD4^+$ T-cell subset.

use of peptide-HLA class I tetrameric complexes with intracellular cytokine staining to assess the functional heterogeneity of $CD8^+$ T cells. These investigators showed that the majority of circulating $CD8^+$ T cells specific for CMV and HIV antigens are functionally active with regard to the secretion of antiviral cytokines in response to antigen, although a subset of tetramer-staining cells was identified that secretes IFN-γ and macrophage inflammatory protein (MIP)-1beta but not TNF-α.

USE OF CYTOKINE FLOW CYTOMETRY TO MONITOR VACCINE-INDUCED IMMUNE RESPONSES

Although new combinations of antiretroviral drugs have improved the quality of life and length of survival of patients with HIV infection, it is clear these

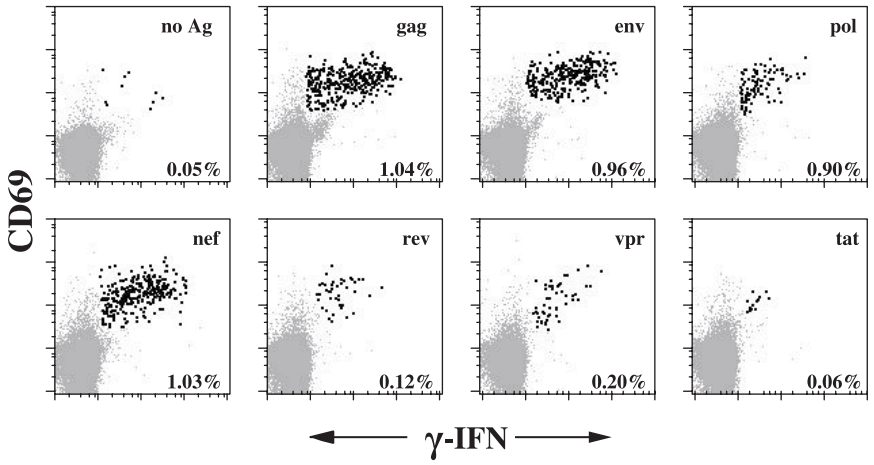

Figure 18.2. Comparison of $CD8^+$ T-cell IFN-γ responses to 15-mer peptide mixes derived from various HIV protein subunits. Represented in this analysis are gag, env, pol, nef, rev, vpr, and tat. Mixes were generated as 15 amino acid sequences overlapping by 11 amino acids.

agents have significant disadvantages. These include associated toxicities, the development of drug resistance, and cost. The development of a successful immunotherapeutic vaccine would represent a significant advance in treatment modality to overcome these problems. In addition, there is a pressing need to develop effective prophylactic HIV vaccines to address the significant HIV infections that plague a number of nonindustrialized countries.

The specific immune mechanisms necessary to elicit HIV vaccine protection remain undefined. Although combination antiviral drug therapy seems to partially reconstitute immunity to microbial antigens, it has not yet demonstrated an effect on enhancing immune responses to HIV antigens in chronic HIV infection (Angel et al., 1998; Autran et al., 1997; Kelleher et al., 1996; Lederman et al., 1998; Rinaldo et al., 1999). In contrast, strong HIV-specific T-helper immune responses to core proteins have been demonstrated in subjects with nonprogressive disease and in patients with primary infection on antiviral drug therapy (Haas et al., 1997; Lisziewicz et al., 1999; Rosenberg et al., 1997; Schwartz et al., 1994). In earlier studies, we examined the cellular cytokine responses to HIV antigens as a consequence of in vivo immunization with a preparation of gp120-depleted whole-killed immunizing antigen (HIV-1). This study demonstrated increases in frequencies of memory $CD4^+$ T cells as a consequence of in vivo immunization with HIV-1 antigen in HIV-1 seropositive subjects placed on long-term HAART therapy (Maino et al., 2000). The observed role of CTL in the containment of AIDS virus replication suggests that

an effective HIV vaccine will require a strong $CD8^+$ T-cell response. Barouch and co-workers recently demonstrated DNA vaccine-induced protective responses against a pathogenic SHIV-89.6 challenge in rhesus monkeys (Barouch et al., 2000). Protection was associated with strong CTL responses (IFN-γ-positive $CD8^+$ T cells) and preserved $CD4^+$ T-cell responses as determined by cytokine flow cytometry. Although the role of CTL seems to be crucial for protective immunity induced by vaccines, and cytokine expression represents a key component of the protective response, the exact function of these cells has yet to be defined. The ability to simultaneously examine multiple parameters makes cytokine (and chemokine) flow cytometry an important tool to further assess functional of responses of $CD8^+$ T cells to both therapeutic and prophylactic HIV vaccine candidates. By combining this technique with class I MHC TCR probes, for example, the functional heterogeneity (cytokine expression) of the antigen-specific $CD8^+$ T-cell response can be addressed (Appay et al., 2000; He et al., 2001).

CONCLUSION

The immune system is the primary target of HIV. The clinical consequences are the results of immune dysfunction and depletion. Within the past 5 years or so, remarkable progress has been made in the understanding of the effects of HIV infection on the dynamics of specific T-cell immunity. Recently, new assays that identify cytokine expression by single cells have been developed that enable the functional identification of antigen-specific T cells. The direct visualization of T-cell effector responses afforded by CFC allows an unparalleled ability to determine the functional potential of phenotypically distinct T-cell subsets, expressing multiple cytokines and chemokines and thus the opportunity to evaluate the participation of these subsets in human immune responses in HIV disease. A number of recent studies using these techniques have begun to address the changes in antigen-specific responses and cytokine alterations caused by antiretroviral therapies and vaccine-induced immune responses. The use of antigen preparations consisting of peptide mixtures has allowed the simultaneous analysis of both $CD4^+$ and $CD8^+$ T-cell cytokine responses to multiple epitopes independent of MHC haplotype and knowledge of T-cell repertoires. Further investigations with these powerful techniques of cell-mediated immunity in HIV disease and the analysis of T-cell responses to vaccines and antiretroviral drugs will be important for the development of new strategies for the treatment of AIDS.

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19

Chimeric Models of SCID Mice Transplanted with Human Cells: The Hu-PBL-SCID Mouse and Its Use in AIDS Research

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INTRODUCTION

SCID (severe combined immunodeficient) mice are characterized by a genetic defect in their DNA repair system that does not allow gene rearrangement of both immunoglobulin and T-cell receptor genes. Because they lack mature B and T cells, SCID mice are profoundly deficient in both humoral and cellular immunity and cannot reject allografts and xenograft (Bosma and Carroll, 1991). Two major chimeric human/SCID mouse models have proved to be of special interest in acquired immunodeficiency syndrome (AIDS) research: 1) the so-called ''hu-PBL-SCID mouse model,'' originally developed by Mosier and co-workers (1988); and 2) the ''SCID-hu model,'' described by McCune and colleagues (1988). Both animal models have been widely used over the last decade for studies on the pathogenesis and therapy of HIV-1 infection. In this chapter, we will review the major characteristics of the hu-PBL-SCID mouse model, which has been extensively used in our laboratory over the last 5 years. The specific features of the SCID-hu model, in which human hematopoie-

tic progenitors contained in fetal liver or bone marrow tissue grafts are co-implanted with fetal thymus tissue to create a chimeric animal (McCune et al., 1988), and its use in AIDS research have recently been reviewed elsewhere (McCune et al., 1997).

In 1988, Mosier and colleagues first reported that human peripheral lymphocytes can stably be engrafted in SCID mice (Mosier et al., 1988). Human peripheral lymphocytes were injected intraperitoneally in SCID mice and persisted in the peritoneal cavity and organs for several months, converting them in the so-called xenochimeric hu-PBL-SCID mice. Since its description, the hu-PBL-SCID mouse model has been widely used to study selected aspects of the human immune response, autoimmunity, and infectious diseases (Duchosal et al., 1990; Mima et al., 1995; Mosier, 1996; Saeki et al., 1992). In particular, xenochimeric mice grafted with human peripheral blood lymphocytes (PBLs) can be successfully infected with HIV as originally reported by Mosier and collegues (Mosier et al., 1991) and several immunological and viral parameters can be easily monitored in these virus-infected chimeric animals.

HU-PBL-SCID MICE: MAJOR TECHNICAL ASPECTS OF THE MODEL

To obtain a hu-PBL-SCID mouse xenochimera, we injected $20–40 \times 10^6$ purified human peripheral blood mononuclear cells into the peritoneum of 3- to 5-wk-old SCID mice (higher numbers may result in a lethal graft versus host disease). The injected cells rapidly (within 24 h after the intraperitoneal injection) spread from the peritoneal cavity, settling in the liver, lymph nodes, the white pulp of the spleen and bone marrow. However, about 1 or 2 months later, the peritoneal cavity remained the major source of human cells that did not migrate to mouse organs, or possibly, that recirculated back to the peritoneum. Cells can be recovered by peritoneal washing with small volumes (e.g., 1 ml) of cold phosphate buffered saline (PBS). After centrifugation, supernatants can be stored at 80°C for cytokine or soluble factors analysis, whereas cells can be pooled with a successive washing with a larger volume of cold PBS. Typically, $1–3 \times 10^6$ total cells are obtainable, consisting of both human and mouse cells. By fluorescence-activated cell sorter (FACS) analysis, according to light scatter properties, human lymphocytes are detected as the higher ''forward light scattered'' cells in a cloud of heterogeneously sized lymphoid cells (Fig. 19.1) and are easily stained by monoclonal antibodies toward CD45, CD3, or CD4 and CD8 human surface markers. Analysis of the percentage of CD4 and CD8 cells is better performed by triple staining, electronically gating CD3 positive cells.

SCID mouse spleens are smaller compared with those of immunocompetent mice, and these organs occasionally reach abnormal size upon reconstitution. However, few human cells can be recovered from spleen disruption, very often accounting for $<1\%$ of the total splenic cell population. On the other hand, lymph nodes are not easily detectable, because they are very small in size and

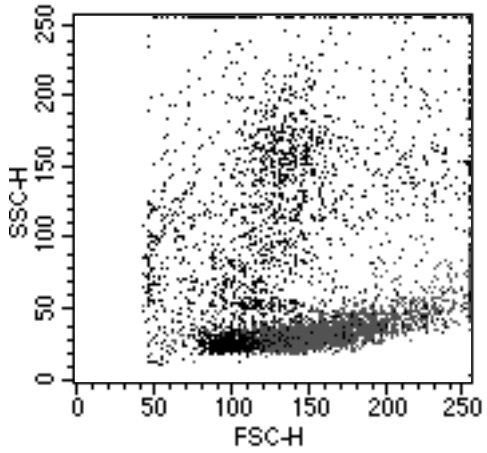

Figure 19.1. Dot plot analysis showing the heterogeneous and peculiar forward and side light scatter properties of peritoneal cells recovered from Hu-PBL-SCID mice at 4 wks after reconstitution. Human cells appear as the relevant lymphoid population evidenced in RI region.

enclosed in mouse fat. It is generally preferable to collect a certain number of mouse lymph nodes and to pool them for subsequent analysis.

In experiments of HIV-1 infection of hu-PBL-SCID-mice, spleen and lymph nodes can generally be used for polymerase chain reaction (PCR) analysis and/or co-cultivation assays with phytohemagglutinin (PHA)-stimulated PBLs to monitor p24 release and recover infectious virus.

RECIPROCAL HOST–GRAFT REACTIONS AND DYNAMICS OF HUMAN PBL ENGRAFTMENT

SCID mice generally do not reject allogeneic or xenogeneic organ grafts and represent a unique model for investigating in vivo the behavior of both normal and neoplastic human cells (Bosma and Carroll, 1991; Mosier, 1996). However, xenogeneic transplantation in SCID mice may fail completely because of the murine natural immune reaction (Zubair et al., 1996). In fact, SCID mice retain fully functioning and highly efficient natural killer (NK) cells, macrophages, and granulocyte compartments (Bosma et al., 1991). Studies aimed at improving human cell engraftment have shown that control of the natural immune reactions through the injection antibodies against either NK cells (Barry et al., 1991; Habu et al., 1981; Lacerda et al., 1996) or granulocyte antigens (Santini et al., 1998) can improve human cell engraftment. We reported that the major reaction of SCID mice to the engraftment of human PBL was a massive granulocyte recruitment into the site of transplantation (Santini et al., 1995). In fact, FACS analysis of the cells recovered from mouse peritoneum 2 h after engraftment revealed an increase in murine $H2K^d$ cells, which were specifically stained

with the anti-polymorphonuclear leukocyte (PMN) antibody RB6-8C5. Likewise, cytospin examination confirmed that neutrophils were the most relevant cell population (55–70% of peritoneal cells), leading to a 70- to 80-fold increase of the number of cells recovered from the peritoneum with respect to uninjected mice. In a few days, thymus, spleen and lymph nodes became infiltrated by murine granulocytes. Notably, daily treatment of SCID mice with antigranulocyte antibody was shown to result in an earlier and more rapid growth of the human lymphoblastoid and monocytoid cell lines CEM and U937 (Lapenta et al., 1997), after their subcutaneous injection and an increase of human PBL persistence in the peritoneal cavity after intraperitoneal inoculation. The granulocyte reaction of SCID mice is not exclusively directed against human cells of hematopoietic origin (Lapenta et al., 1997; Santini et al., 1995). In fact, we have recently reported that murine granulocytes infiltrate and delimit human tumors transplanted in SCID mice and that treatment with antimurine granulocyte antibody markedly improves the growth of human tumor cell lines of different origin, through suppression of the host granulocyte reaction (Lozupone et al., 2000).

Reverse transcriptuse (RT)-PCR analysis of peritoneal cells reveals that a wide array of murine cytokines such as interleukin (IL)-1, IL-4, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ (with the exception of IL-2) is rapidly induced after transplantation of human PBL into SCID mice (Santini et al., 1995). The study of the kinetics of murine cytokine production, as evaluated by enzyme-linked immunosorkent assay (ELISA) of peritoneal washings from hu-PBL-SCID mice reveals a peak of TNF-α and IL-12 occurring at 2 h after engraftment, followed by a long lasting induction of IL-6 and IFN-γ, which could be detected in mouse sera for up to 2 wks (Santini et al., 1995). As a result of human PBL transfer, an impressive increase in murine leukopoiesis can take place in both bone marrow and spleen, accompanied by an expansion of the red pulp cells, whereas minimal foci of extramedullary hematopoiesis are occasionally observed in the liver and peripheral lymph nodes. FACS analysis of thymocytes from hu-PBL-SCID mice reveals the presence of $CD4^+/CD8^+/CD3^-$ and $CD8^+/CD3^-$ cells between 12 and 14 wks, which cannot normally be found in unreconstituted mice (Santini et al., 1995).

Human cells residing in hu-PBL-SCID mice are mainly mature CD4 and CD8 cells. However, CD4 cells in the peritoneal cavity progressively decline until CD8 cells naturally predominate. Few NK cells and monocytes are recovered from mouse peritoneum as soon as 24 h after cell graft and rapidly become undetectable in peritoneal cell washings, although small adherent foci of human macrophages can be detected in peritoneal serosa (our unpublished observations). Even though human B cells rapidly become undetectable in peritoneal washings, they settle down in mouse organs and undergo a CD4-dependent conversion into immunoglobulin (Ig)-producing plasma cells. Thus, a progressive increase in the detection of human IgG, IgM, and IgA, ranging from 100 to 500 μg/ml in 2 wks, can be observed in the sera of xenochimeras (Rizza et al., 1996). Humoral response is preceded by an early and generalized

activation of human lymphocytes. In fact, as early as 2–24 h after cell injection, the mRNA accumulation of a wide spectrum of human cytokines (IL-2, IFN-γ, IL-4, IL-10, TNF-α, TNF-β) is readily detectable in peritoneal cells, together with increased levels of IL-2R(p55) and ICAM-1 mRNA. Soluble IL-2R and ICAM-1 can be consistently detected in mouse sera and peritoneal lavages for several weeks. Notably, an impressive up-regulation of lymphocyte activation markers can be observed. In particular, CD69 is rapidly induced, whereas $CD25^+$ cells increase during the first week and then decline, paralleling an ongoing rise in the percentage of human leukocyte antigen (HLA)-DR and CD45RO cells. At 2 wks after human cell transfer, the majority of the lymphocytes in the peritoneal environment express the memory isoform CD45RO. Human cell spread can be easily monitored and documented by PCR analysis of human DNA in mouse tissues. In fact, human HLA-DQα sequences can be detected in various organs of xenochimeric mice as early as 2 h after reconstitution up to several weeks after human PBL injection (Rizza et al., 1996).

In the light of the results summarized above, the reciprocal activation between human and mouse cells appears to be a dynamic scenario, in which the human immune cell response and repertoire can be progressively shaped by the strong reaction toward the murine major histocompatibility complex (MHC) and partly killed by mouse reaction. This process can favor the escape of human cells from mouse peritoneum as a result of vascular and mesothelium injuries and cytokine-induced derangement of cellular traffic through murine endothelium.

HU-PBL-SCID MICE AND HIV INFECTION

Taking into account the scenario of events occurring in hu-PBL-SCID mice, we have modified the original model of HIV infection of hu-PBL-SCID mice developed by Mosier and co-workers based on the virus challenge 2 wks after transplantation of human PBLs (Mosier et al., 1991). Human T lymphocytes turn into CD45RO memory cells within 2–3 wks after transplantation (Fig. 19.2) and can become mostly anergic (Tary-Lehmann and Saxon, 1992; Tary-Lehmann et al., 1995). In contrast, soon after reconstitution, human T cells are highly activated, expressing the surface activation marker CD69, followed by HLA-DR and CD25 expression (Fig. 19.2). Thus, we infected hu-PBL-SCID mice 2 h after hu-PBL injection with the X4 laboratory strain IIIB, as compared with the classical model of HIV infection at 2 wks after reconstitution of SCID mice. A marked $CD4^+$ T-cell depletion and a significant reduction in IgA, IgM, and sIL-2R serum levels and sICAM-1 in peritoneal washings were observed in the xenochimeras infected with HIV-1 at 2 h, whereas the immune functions of hu-PBL-SCID mice infected at 2 wks were almost comparable to that of uninfected controls. Of interest, the IgM serum levels in infected animals directly correlated with the levels of CD4 found in the peritoneal cavity (Fig. 19.2).

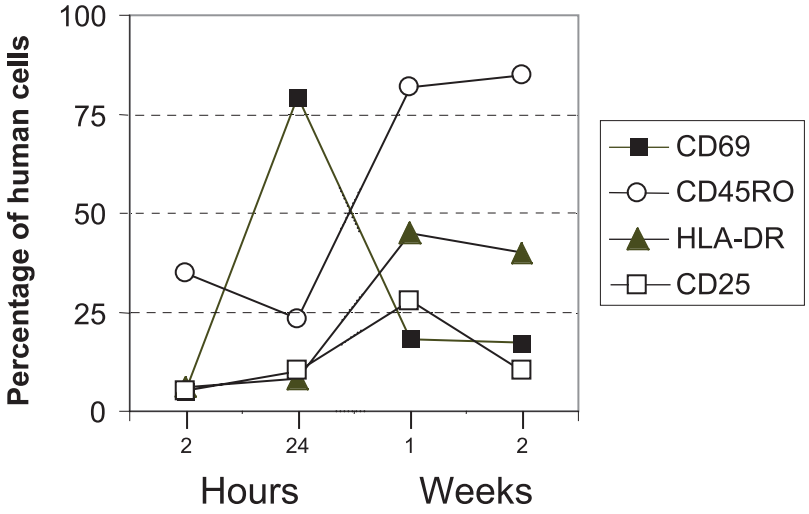

Figure 19.2. FACS analysis of T-cell surface markers. Cells were collected from peritoneal cavity at different time points after engraftment, as reported on figure, and stained with the different monoclonal antibodies.

Surprisingly, the generalized impairment of immune functions detected in the xenochimeras infected at early times was accompanied by the selective up-regulation of the expression of some cytokines (IL-4, IL-5, and IL-10) as revealed by RT-PCR. This was reminescent of the T-helper type 2 (Th2) profile observed in HIV patients experiencing rapid progression of the disease and a worse prognosis. The switch of Th1 into the Th2 profile of cytokine production has been also suggested to account for the higher rate of $CD4^+$ T-cell apotosis seen in these patients (Clerici et al., 1994). Notably, it has been suggested that the state of activation of immune cells affects the rate of HIV replication and disease progression in HIV-infected individuals (Bentwich et al., 1995; Stevenson et al., 1990; Wachter et al., 1996). In particular, it has been argued that the chronic state of immune activation (due to the parasitic and bacterial diseases) is the major cause of the impressive prevalence and incidence of HIV infection in Africa as well as of the rapid progression to AIDS in the infected patients (Bentwich et al., 1995). This is highly consistent with data obtained in simian immunodeficiency virus (SIV)-infected macaques, where hyperimmune activation has been shown to accelerate disease progression (Folks et al., 1997). Thus, the ensemble of experimental data obtained in our modified SCID model support the concept of the important role of acute or chronic immune activation in natural infection and in rendering the host more receptive to HIV and more sensitive to its pathologic effects on the immune functions.

In 1993, Mosier and colleagues published the unexpected finding that macrophage-tropic viruses induced a more rapid loss of CD4 T cells in the hu-PBL-SCID mice than did the T-cell-tropic isolates, which possessed a high in-

trinsic replication rate and a syncytia-inducing phenotype and which induced a rapid cytopathic effect in vitro (Mosier et al., 1993). Thus, we have recently compared HIV-1 monocytotropic R5 and lymphotropic X4 strains of HIV-1 for their infection of hu-PBL-SCID mice at different times from reconstitution, for the extent of viral replication, the rate of CD4 T-cell depletion, and the alteration of human immune parameters (Fais et al., 1999). Measurement of proviral load in spleen and lymph nodes by PCR and the rescue of infectious HIV-1 by cocultivation of cells recovered from peritoneum and organs with PHA-activated PBLs showed a good correlation between the extent of HIV-1 infection and the CD4 depletion rate. In fact, distinct patterns of effects were detected, depending on the time of infection of the reconstituted SCID mice. A comparable level of infection was found when hu-PBL-SCID mice were infected with either the R5 SF162 or the X4 IIIB HIV-1 strains at 2 h after reconstitution. Challenge with the R5 strain resulted in high levels of virus replication and in a marked CD4 T-cell depletion together with a significant drop in IgM and soluble factors levels at both 2 h and 2 wks after human cell grafting (Fig. 19.3). In contrast, the X4 virus induced a very low rate of CD4 depletion and viral replication, with limited viral spread to mouse spleen and lymph nodes, when injected 2 wks after reconstitution. Because HIV-1 cell tropism is strictly linked to determinants found on the viral envelope glycoprotein gp120, we compared the parental X4 strain SF2 and the R5 strain SF162 with chimeric recombinant viruses exhibiting reciprocal substitutions of the *env* gene on the backbone of the parental SF2 and SF162 genomes. The analysis of xenochimeric mice challenged with the four viruses showed that the *env* gene played a major role in determing the pattern of in vivo T-cell depletion and immune alterations. In fact, the parental SF162 and the chimeric SF2 harboring the SF162 *env* gene generated comparable high viral burden and induced a severe T-cell depletion when injected at both early (2 h) and late (2 wks) time points after reconstitution, whereas both the parental SF2 and the chimeric SF162 carrying SF2 *env* gene behaved as the prototypic X4 IIIB virus, causing high levels of viral replication and severe CD4 T-cell depletion only when injected 2 h after reconstitution of SCID mice (Fig. 19.4). These results emphasize the major role played by the *env* gene in determining the pathogenic potential of distinct HIV-1 strains in the hu-PBL-SCID model. It is of interest to consider that this phenomenon was consistent with that observed in simian-human immunodeficiency virus (SHIV)-infected macaques (Karlsson et al., 1998). In this model, the rate of T-cell depletion was determined by single amino acid substitutions in the *Env* ectodomain, which conferred increased chemokine receptor binding and enhanced membrane fusogenic activity. Of interest, the SCID mouse environment influences the differentiation of primary T lymphocytes and lymphoblastoid cell lines, modulating phenotype and chemokine receptor expression. CXCR4 is mostly expressed on naive CD4 T cells, whereas CCR5 is generally found on memory T cells (Bleul et al., 1997). In fact, CCR5 expression on human CD4 T cells is detectable as early as 24–48 h after PBL injection into SCID mice and increases considerably after 1–2 wks, together with a decrease

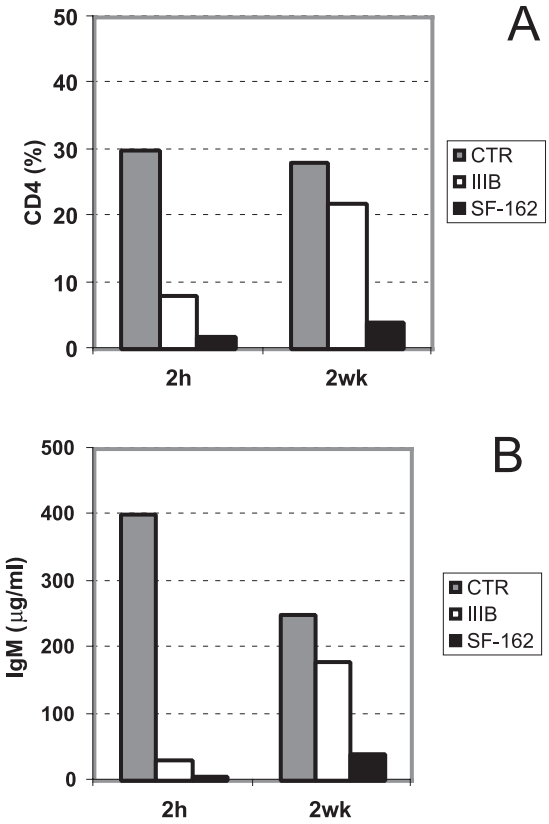

Figure 19.3. (**A**) HIV-1-induced T-cell depletion. Hu-PBL-SCID mice were challenged with HIV-1 (either IIIB or SF-162 strain) at 2 h or 2 wks after engraftment and sacrificed 4 wks later. (**B**) Effect of HIV-1 infection on human IgM serum levels. Note that the levels of human IgM directly correlate with the levels of human CD4 T cells.

in CXCR4 expression. The latter phenomenon can explain, at least in part, the reduced susceptibility of hu-PBL-SCID mice to X4 viruses when injected at this time. Notably, in vivo–induced alterations of the human T-cell phenotype are not a unique characteristic of primary T cells. In fact, we had shown that subcutaneously injected CEM cells acquire a memory-like phenotype, up-regulating CD45RO, CCR2, CCR3, and CCR5, whose expression is associated with a high susceptibility to the in vivo infection with the HIV-1 monocytotropic strain SF162 and with a series of monocytotropic clinical isolates, which cannot infect CEM cells in vitro (Lapenta et al., 1998). The susceptibility of CEM cells transplanted into SCID mice to HIV infection is selectively inhibited by the β-chemokines such as RANTES, macrophage inflammatory protein (MIP)-1α and MIP-1β (the natural CCR5 ligands), and is progressively lost upon long-term culture after explantation from xenochimeras. These findings were highly

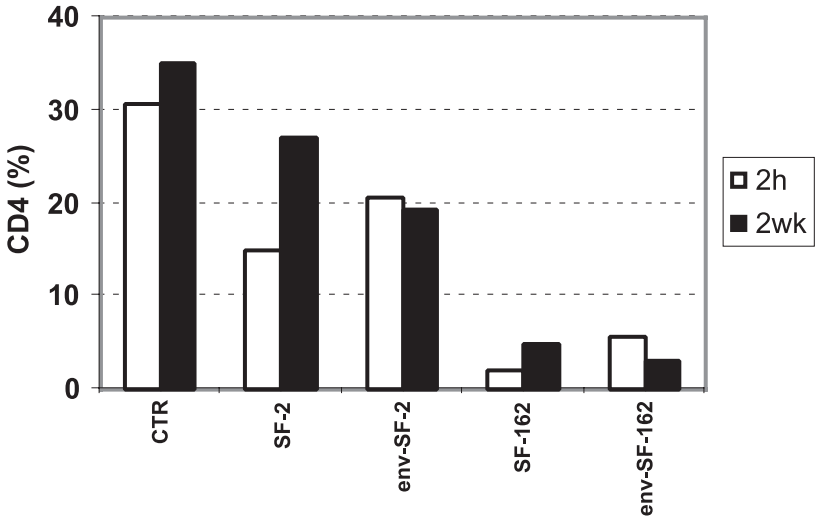

Figure 19.4. Effect of HIV-1 infection on CD4 T cell levels. Hu-PBL-SCID mice were challenged with SF-2, SF-162, and chimeric recombinant viruses exhibiting reciprocal substitutions of the *env* gene on the backbone of parental SF-2 and SF-162 genomes. env-SF-162 indicates the recombinant SF-2 virus carrying the SF-162 *env* region, whereas env-SF-2 indicates the recombinant SF-162 virus carrying the SF-2 *env* region. Xenochimeras were challenged with the different viral strains at 2 h or 2 wks after engraftment and sacrificed 4 wks later.

consistent with the acquisition of a memory phenotype by CEM cells during their growth in SCID mice (Lapenta et al., 1998).

TESTING OF ANTIVIRAL SUBSTANCES AND STRATEGIES

Any substance, before its clinical use, has to be tested in an animal model, in which drug bioavailability, metabolism, clearance, toxicity, and antiviral efficacy can be studied. Although hu-PBL-SCID mice proved to be valuable tools for investigating some aspects of human immunodeficiency virus (HIV) infection and AIDS pathogenesis, their current use for antiviral screening has been hampered by two major limitations: i) the restriction of HIV-1 infection to a limited number of human cells, resulting in poor levels of viremia; and ii) the relatively short time of persistence of virus infection. In this regard, human cell lines grafted into SCID mice may offer new opportunities for the preclinical testing of antiviral drugs and therapeutic strategies, especially because of their practical advantages and potential implementation. We recently reported that different lymphoblastoid and monocytoid cell lines can be injected into SCID mice to support HIV-1 infection. Anti-mouse α/β IFN or anti-PMN antibody treatment can be performed to enhance tumor growth, resulting in a high level of engraftment. HIV-1 infection of the graft results in multiple virus replication

cycles leading to an impressive p24 antigenemia (up to 4×10^3 pg/ml), very high levels of circulating infectious virus, and up to 5×10^7/ml copies of viral RNA in mouse sera. Thus, the high reproducibility, well-defined kinetics of virus production, and long lasting in vivo persistence of virus-infected cells, together with low cost characteristics, render this model particularly suitable for some studies of anti-HIV therapy (Lapenta et al., 1997). In a prototypic study, we observed a complete inhibition of both p24 and viral load in the sera of xenochimeric mice treated with AZT (480 mg/kg/day), while at the tumor site a marked reduction of infected cells and of the level of virus expression could be evaluated (Lapenta et al., 1997). This model was also utilized for studying the efficacy of IFN-α treatment in SCID mice co-injected with the monocytoid cell line U937 and HIV-1. IFN-α was highly effective in inducing suppression of HIV-1 infection in this model. Thus, we evaluated the possible effects of low levels of endogenous IFN, produced locally as a consequence of viral infection (Lapenta et al., 1999). We used genetically modified U937 cells expressing the Tat-transactivable IFN-α2 gene transplanted into SCID mice and infected with HIV-1. Low levels of locally produced IFN-α not only proved to effectively inhibit acute HIV infection and replication, but also conferred a strong protection to bystander parental U937 cells coinjected with genetically modified U937 cells (Lapenta et al., 1999). However, if chimeric models of SCID mice transplanted with human cell lines may have some practical advantages for a rapid evaluation of some antiviral therapies, the hu-PBL-SCID model has the unique characteristic of allowing evaluation of the impact of antiviral interventions on human immune cell functions. As an example, we found that the potent antiviral activity of IFN-α in hu-PBL-SCID mice infected with HIV-1 is associated with the development of a primary humoral immune response to HIV, which can participate in the cytokine-induced suppression of virus replication (Lapenta et al., 1999).

CONCLUSION

Since its development, the Hu-PBL-SCID mouse model and its wide range of minor and major modifications or adaptations have proved to be very useful as valuable in vivo approaches for shedding light on several aspects of the pathogenesis of HIV-1 infection. However, its use in the evaluation of the antiviral activity of anti-HIV interventions has been partially hampered by the intrinsic features of xenochimeric mice, which exhibit only a limited number of human immune functions and lack physiologic mechanisms of immune cell turnover and replenishment. In fact, once HIV-1 infection has been established, it rapidly spreads to uninfected cells. Therefore, it becomes quite difficult to block or reverse the infection process in this animal model, thus resulting in a limitation of its use in drug testing and vaccine development. In spite of this, taking into account these limitations and finely adjusting/adapting the model to address

selected issues, the hu-PBL-SCID mouse model offers unique opportunities for in vivo study of the efficacy of novel immune interventions against HIV and, more generally, against viral infections. In particular, even though the feasibility of a current use of hu-PBL-SCID mice in vaccine research is still a matter of debate, we believe that hu-PBL-SCID mice can serve as very versatile and practical animal models for investigating not only passive but also active immunity against HIV-1 infection. In general, studies on active immunization have been hampered by difficulties in obtaining a primary human immune response in the xenochimeric mice. The major limitations for developing a human immune response in this model seem to be due to a limited human immune repertoire, which is rapidly shaped by murine environment (Tary-Lehmann et al., 1994), the lack of appropriate human antigen-presenting cells (APCs) and, finally, to the onset of T-cell anergy (Tary-Lehmann and Saxon, 1992). Despite this, the generation of a primary human immune response in xenochimeric mice has been reported in a few studies using different approaches to enhance antigen presentation by using adjuvants, liposomes, and antigen-pulsed dendritic cells (Coccia et al., 1998; Walker et al., 1995, 1996), or by providing a suitable microenvironment by engrafting SCID mice with human skin and autologous PBLs (Dehelm et al., 1998). Recently, we postulated that an efficient immune response could be elicited in hu-PBL-SCID mice, provided that appropriate antigen presentation could occur in vivo as a result of transfusion of autologous APCs soon after reconstitution with hu-PBL, before the irreversible selection of xenoreactive human T-cell clones takes place. We recently observed that infusion of autologous antigen-pulsed macrophages within a few days of reconstitution, followed by a boost immunization after 7 days, generated a good antibody response (unpublished observations). Moreover, cultured autologous dendritic cells, generated from monocytes after exposure to granulocyte-macrophage colony-stimulating factors (GM-CSF) and type I IFN pulsed with inactivated HIV-1 induced the generation of a potent primary immune response toward HIV-1 after injection in hu-PBL-SCID mice, as evaluated by the detection of specific human antibodies against the spectrum of viral proteins. At 7 days after primary immunization, human antibodies proved to be mostly IgM, whereas HIV-1-specific IgG1 antibodies were detected after boosting (Santini et al., 2000). Notably, the antibodies detected in the sera of mice injected with dendritic cells generated in the presence of IFN exhibited a neutralizing activity in vitro against HIV-1 (our unpublished observations).

In conclusion, the SCID mouse models have considerably contributed to generate the current knowledge on the pathogenesis and therapy of HIV-1 infection over the last decade. These models, which are far more practical and less expensive than other animal models for AIDS research, represent unique approaches for studying in vivo the interactions between HIV and human immune cells. Even though a considerable amount of information on the pathogenesis of HIV-1 infection has already been achieved by the use of hu-PBL-SCID mice, we believe that the versatile characteristics of this practical animal model and

the multiple potential approaches for its implementation offer novel opportunities for its use in the evaluation of immune interventions against AIDS and, more generally, in vaccine research.

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20

Immune Reconstitution of the CD4 T-Cell Compartment in HIV Infection

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INTRODUCTION

The infection of the CD4 T-cell compartment by the human imunodeficiency virus (HIV) leads to a progressive CD4 cell depletion and to an inflammatory disease of the immune system that eventually causes major cellular immune defects with severe infectious and tumoral opportunistic events. Flow cytometry has helped describe a number of cellular alterations apart from the CD4 cell depletion, quantification of which is the routine marker used to measure progression of the disease. The introduction of highly active antiretroviral therapy (HAART), combining inhibitors of the HIV-1 reverse transcriptase and protease, has dramatically modified the course of the HIV infection during the past 4 years (Colier et al. 1996; Gulick et al., 1997; Hammer et al. 1997; Katznstein et al., 1996). Despite some controversies about the extent to which the immune system can normalize, it is now generally admitted that immune reconstitution can be obtained and can confer host protection against opportunistic events (Autran, et al. 1997; Li, et al. 1998; Pakker et al., 1998; Lederman et al., 1998). The best hallmark of such immune restoration is the progressive reconstitution of a normal or subnormal CD4 cell compartment and the massive decline in the mortality and morbidity related to acquired immunodeficiency syndrome (AIDS)

that has been registered in all industrialized countries (Buchbinder et al., 1999; Hogg et al., 1997; Pallela et al., 1998). The immune reconstitution obtained with these antiretroviral drugs, even if incomplete, has produced definitive evidence for the central role played by HIV itself in the massive alterations of the immune system. Intense publishing activity followed the first descriptions of immune reconstitution with antiretroviral therapy, illustrating the enthusiasm and the controversies raised by these observations.

We will review the major quantitative, qualitative, and functional characteristics of the CD4 T-cell reconstitution together with its limitations and the new insights that recently developed flow cytometry methods have brought to the understanding of such CD4 T-cell reconstitution.

THE CD4 T CELL COMPARTMENT IN THE PRE-HAART ERA OF THE HIV INFECTION

The CD4 T-cell depletion results from a direct pathogenicity of HIV and/or from the cytolysis mediated by cytotoxic T lymphocytes (CTL) against the infected CD4 cells that actively replicate the virus. "Innocent" non-HIV-infected CD4 T cells also die in excess during the course of the natural infection due to a major activation of the immune system that results in an enhanced activation-induced cell death. The peripheral blood CD4 cells reflect, however, only 2% of the total body CD4 compartment. The severity of the CD4 cell loss in lymphoid tissues might not be as severe, however, as suggested by peripheral blood counts, because CD4 cells might be sequestered in the lymphoid organs during the natural course of the disease (Rosok et al., 1996). Losses mainly affect the activated memory CD4 T cells, which can be operationally defined by the cell surface co-expression of the CD45RO isoform. Various markers of T-cell activation, such as CD25, human leukocyte antigen (HLA)-DR, CD38, or Fas (Badley et al., 1998; Giorgi et al., 1997) are abnormally expressed on those cells as a correlate of disease progression and plasma HIV load, whereas some markers associated with T-cell competence and T-helper differentiation, such as CD28 and CD7 (Borthwick et al., 1994; Autran 1996), have a decreased expression. Memory T-cell function defects are assessed by a progressive loss in proliferative capacity, interleukin (IL)-2 production, and T-helper-1 cell reactivity against various recall antigens and opportunistic pathogens (Clerici et al., 1989; Lane et al., 1985). A major controversy was raised as to whether the turnover of peripheral mature $CD4^+$ T cells was enhanced (Hellerstein and McCune, 1997; Hellerstein et al., 1998; Ho et al., 1995; Perelson et al., 1996; Wei et al., 1995; Wolthers et al., 1996). According to the tap and drain hypothesis (Ho et al., 1995; Perelson et al., 1996), an increased CD4 cell proliferation should compensate cell losses at early stages of the disease and should become detectable during the rapid increase of CD4 counts that follows therapeutic reduction of viral replication. Flow cytometry analyses helped provide rough data to support or reject such a hypothesis. An increased expression of

intracellular markers associated with cell cycle, such as Ki67, was reported in CD4 T cells as an inverse correlation of the CD4 cell depletion (Fleury et al., 1998; Lempicki et al., 2000; Orendi et al., 1998; Sachsenberg et al., 1998; Tenner-Racz et al., 1998). Little evidence could support, however, the hypothesis of a high replacement rate. Indeed, telomeres were shown to maintain a subnormal length in CD4 T cells (Wolthers et al., 1996). We demonstrated that Ki67 was not associated with mitosis in memory CD4 T cells from HIV-infected patients but only with entry in the G1 phase of the cell cycle and T-helper cell activity (Combadière et al., 2000). Finally, no increased incorporation of BrdU could be detected by ex vivo flow cytometry in CD4 T cells from HIV-infected patients (Tissot et al., 1998). Thus, the increased expression of Ki67 reflects massive mobilization of activated memory or effector CD4 T cells during a chronic virus infection rather than a massive enhancement of the CD4 cell turnover (Combadière et al., 2000). Nevertheless, increased incorporation of deuterated glucose in CD4 T cells could be related to a three-fold increase of the CD4 T cell turnover (Hellerstein et al., 1999), which was compatible with Ki67 and telomere data (Fleury et al., 1998). In addition, bone marrow progenitor production (Marandin et al., 1996) and thymic T-cell production (Bonyhadi et al., 1993) were shown to decrease during HIV disease progression, although the thymic mass does not decrease significantly (McCune et al., 1998). This low replacement rate together with an enhanced conversion rate of naive-to-memory CD4 T cells due to the intense immune activation ultimately leads to naive T cell depletion (Roederer 96, Herzenberg 2000).

Normalizing these phenotypes and functions with antiretroviral drugs thus represented a major challenge to the medical and scientific community. Flow cytometry helped to rapidly demonstrate the capacity of the CD4 T-cell compartment to reconstitute in HIV-infected patients together with the kinetics and mechanisms of such reconstitution.

THE DYNAMICS OF CD4 CELL RECONSTITUTION: A TWO-PHASE PROCESS

New antiretroviral drug regimens combining inhibitors of two HIV enzymes, the reverse transcriptase (RTI) and the protease (PI) opened in 1995 a new era by inducing major and stable reduction in viral load accompanied by sustained CD4 cell increases (Collier et al., 1996; Gulick et al., 1997; Hammer et al., 1996; Katzenstein et al., 1996) that had never been previously reported with mono- or bi-therapy with RTI. Such changes immediately raised the question of immune restoration and its mechanisms.

Our group conducted, in 1996, the first study evaluating the consequences of HAART on the various T-cell alterations briefly summarized above (Autran et al., 1997). Patients, naive of any antiretroviral drugs, were treated with one PI and two nucleoside analogs at a late stage of the CD4 depletion with CD4 counts $<250/mm^3$. A very early increase in CD4 counts was characterized

by strong slopes of peripheral blood CD4 T cells of 1–5 CD4 cells/mm^3/day during the first 2 months of treatment (Fig. 20.1*A*) concurrently with a rapid and major reduction in virus load of 1 or 2 log of magnitude (Fig. 20.1*B*). Various other studies confirmed those first results, both on peripheral blood cells (Lederman et al., 1998; Pakker et al., 1998; Pontesilli et al., 1999; Rinaldo et al., 1999) and on lymph nodes (Bucy et al., 1999; Cavert et al., 1997; Fleury et al., 1998; Sachsenberg et al., 1998; Tenner-Racz et al., 1998; Zanussi et al., 1999). Such findings suggested that the CD4 and CD8 T cells had been previously recruited in lymphoid tissues at the time of active virus replication where they had been sequestered in an altered cytokine and chemokine milieu (Cheynier et al., 1994; Rosok et al., 1996). The arrest of local virus production (Cavert et al., 1997; Patki et al., 1999; Wong et al., 1997) in lymphoid tissues should allow the sequestered T cells to recirculate (Bucy et al., 1999), thereby increasing peripheral blood cell counts. Similar phenomena are observed at any stage of the CD4 cell depletion, that is, from primary infection to full-blown AIDS (Carcelain et al., 1999). However, such CD4 cell mobilization substantially increases peripheral blood cell numbers, mostly in patients with low CD4 counts. Thus, no massive gain in the total CD4 cell compartment occurs at this early phase of immune reconstitution.

What are the factors influencing such early rises in CD4 counts? We demonstrated that the first-phase CD4$^+$ T-cell slope is positively correlated with the baseline CD4 count, and even more strongly but negatively correlated with the slope of CD4$^+$ T-cell decline during the year prior to PI initiation (Renaud et al., 1999). This relation with prior CD4$^+$ T-cell decline was also observed among the patients with a prolonged history of major CD4$^+$ T-cell depletion ($<$50/mm^3) and those with higher CD4 counts and slow disease progression before study entry (data not shown). On the other hand, age and amplitude of viral load reduction over a 2-month period had low effects on the early CD4$^+$ T-cell recovery, whereas baseline viral load values had no significant impact (Renaud et al., 1999).

The kinetics of the CD4 cell expansion are usually reduced after the second or third month of treatment (Fig. 20.1*B*) (Autran et al., 1997; Connick et al., 2000; Fleury et al., 2000; Pakker et al., 1998). The slopes of CD4 recovery are usually 1 log lower than during the first 2 months. This second phase of immune reconstitution seems to be strongly correlated with the magnitude of the plasma viral load reduction and its stability over time (Fig. 20.1*B*) (Connick et al., 2000; Renaud et al., 1999). Indeed, a 4 log reduction in the plasma viral load can be associated with a 40% increment slope during the 2 years following HAART initiation whereas a 1 log viral load reduction is usually associated with a 5% slope only (Fig. 20.1*B*). Thus, stable control of the HIV replication allows a steady and continuous increase in CD4 counts. A variation of the mean viral load of -1 $\log_{10}$ HIV-RNA copies/ml between month 2 and month 24 in two otherwise identical patients meant that their second-phase CD4$^+$ T-cell slope differed by $+0.11$ CD4/mm^3/day. These findings suggest that, when

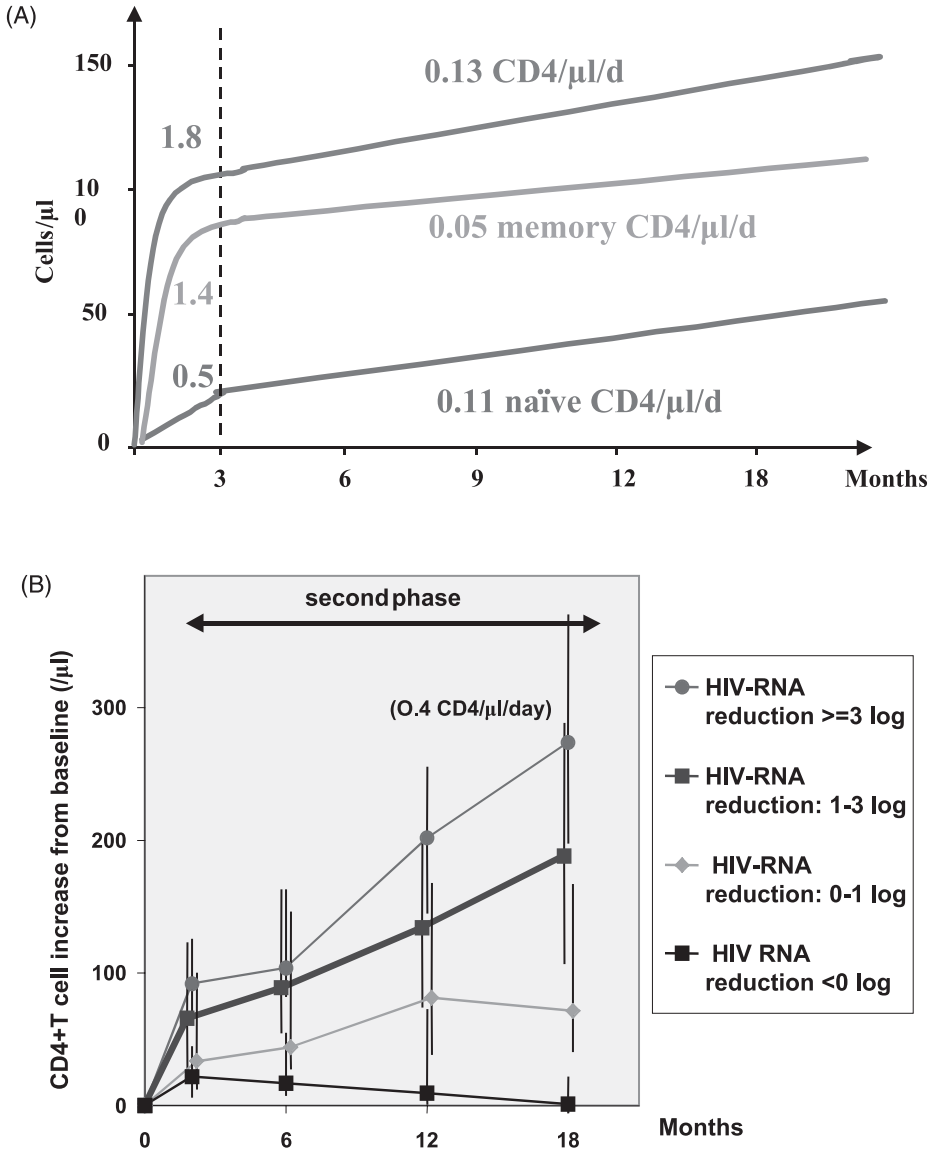

Figure 20.1. Kinetics and mechanisms of CD4 T-cell reconstitution after HAART initiation during late disease. (**A**) An initial increase, usually rapid and large, represents redistribution of memory CD4 T cells, and is followed by a second, slow but steady increase that reflects regeneration of naive CD4 T cells. The slopes are calculated from data published in Autran et al., 1997 and Li et al., 1998. (**B**) The slopes of CD4 increase over the long term depend on the magnitude of the viral load reduction (calculated from Renaud et al., 1999).

the redistribution phenomenon is over, the immune system is repopulated at the same pace in most cases, whatever the severity of the immune alterations that had taken place before introduction of HAART.

Such progressive and virus-dependent reconstitution brings the CD4 counts above the symbolic threshold of 200/mm^3 within 12–24 months in very severe stages of the disease, thus ensuring protection against opportunistic pathogens. Normal or subnormal CD4 values can then be reached within 2–4 years, depending first on the disease stage at which treatment had been initiated and second on the persistent control of the virus replication (Fleury et al., 2000; Mezzaroma et al., 1999; Renaud et al., 1999; Schooley, 1999; Weiss). Thus, most protection will be more rapidly attained when HAART is introduced at early stages than at end stages of the CD4 cell depletion (Miller et al., 1999).

In some patients, however, CD4 count reconstitution seems to be independent of virus control. This phenomenon was first reported in a Swiss cohort (Kaufman et al., 1998) followed by several groups (Levitz, 1998; Piketty et al., 1998). We observed similar phenomena in a group of 320 patients followed in our institution (Renaud et al., 1999). Two different paradoxical patterns were detected at 2 months of therapy. Paradoxical CD4 nonresponders were defined as having a viral load reduction >1 $\log_{10}$ but no CD4$^+$ T-cell increase from baseline. In these cases (8%), the median slope of initial CD4 recovery was -0.26 CD4$^+$ T cells/mm^3/day. On the other hand, paradoxical CD4 responders at month 2 were defined as having a lack of significant reduction in HIV-RNA copies/ml (<0.5 $\log_{10}$) but a CD4 count increase from baseline >50 CD4$^+$ cells/mm^3 (which was the median CD4 gain at month 2 for all study patients). In these cases (7%), the median first-phase slope was $+1.5$ CD4$^+$ T cells/mm^3/day. The low influence of viral load reduction during the first phase of rapid CD4 increase and its strong influence over the long term explain the paradoxical CD4 responses reported by others. Indeed, a simple mathematical model helps explain how a minimal virus load reduction is enough to generate some redistribution from the tissues (Drusano and Stein, 1998). However, the absence of significant CD4 decline observed over the long term in paradoxical CD4 responders still contrasts with the lack of virus control and cannot be fully explained by the factors described herein. Although we could not isolate age as an important parameter, it certainly plays a role in delaying or impairing thymic regeneration (McCune et al., 1998, 2000; Smith et al., 2000)

NAIVE AND MEMORY CD4 T CELLS ARE THE ACTORS OF THE CD4 CELL RECONSTITUTION

Simple flow cytometry analyses helped demonstrate the mechanisms of such CD4 T-cell reconstitution (Fig. 20.1*A*). In our first study, the rapid early wave of CD4 T cells was shown to be mostly composed of memory CD4 T cells bearing the CD45RO isoform. Pakker and colleagues confirmed this early rise in memory T cells, supporting the hypothesis of a cell redistribution from the

tissues (Pakker et al., 1998). We demonstrated a lack of propidium iodide staining in those cells. Other groups also reported a significant decrease in cell cycle markers in those memory CD4 cells (Sachsenberg et al., 1998; Tenner-Racz et al., 1998; Fleury, et al., 1998). In contrast, the memory CD4 T cells that arise during the 2 wks following treatment initiation displayed early activation markers such as CD25, suggesting again a rapid mobilization toward the peripheral blood of the activated T cells that had been previously recruited in the tissues (Rosok et al., 1996).

The second slower phase of CD4 cell reconstitution might involve some T-cell proliferation, as suggested by the in vivo measurement of deuterated glucose incorporation (Hellerstein et al., 1999) or the Ki67 intracellular expression (Fleury et al., 2000). Regeneration of naive T cell was shown in our initial study to play the major role in the second phase of the CD4 subset reconstitution. Indeed, naive CD4 T cells significantly increased both in proportions and absolute numbers after the third month of treatment in patients treated at late stages of CD4 cell depletion. These first findings were confirmed by all subsequent studies (Lederman et al., 1998; Li, et al., 1998; Pakker et al., 1998; Pontesilli et al., 1999). When treatment is introduced, however, at earlier stages of the disease, or even at primary infection (Carcelain, et al., 1999), slopes of naive CD4 cell recovery are indistinguishable from the slopes of memory CD4 T cell, with a rapid and early increase of naive CD4 T cells, indicating that those naive T cells can be explained as the memory T cells, to some sequestration/desequestration phenomenon (Fig. 20.2). Again, whereas the intensity of the naive cell depletion is positively correlated to the CD4 counts at initiation of treatment, the kinetics of the naive cell reconstitution over the long term are a function of the virus control rather than of the disease stage or of the CD4 depletion (Fig. 20.3). After the first 3–6 months of treatment, the slopes of naive cell recovery appear to be parallel, independent of the disease stage, suggesting a very conserved mode of replenishment of the CD4 compartment. (Fig. 20.2). Naive cell reconstitution is thus the main component of the total CD4 cell reconstitution in the long term.

Such increases in naive T cells were the major surprise of these studies when one considers the dogma of a thymic involution in adults that is derived from observations of a very late naive T-cell regeneration after chemotherapy for hematological malignancies in adults (Mackall et al., 1993, 1995). These flow cytometry observations immediately raised a controversy over the origin of these so-called naive CD4 T cells. Indeed, some memory T cells can revert their CD45 isoform from RO to RA (Bell and Sparshott, 1990), although such phenomena occur mostly in the CD8 compartment. To overcome such a difficulty, we assessed the naive cell status by demonstrating the coexpression of the CD45RA and CD62 L-selectin (Autran et al., 1997), the latter molecule allowing naive cell penetration in lymph nodes (Picker et al., 1993). We also checked that the $CD45RA^{+}62L^{+}CD4^{+}$ cells had functional characteristics of naive T cells that had not previously encountered antigens and are unable to proliferate against recall antigens nor to produce effector cytokines (Fig. 20.4). Our first

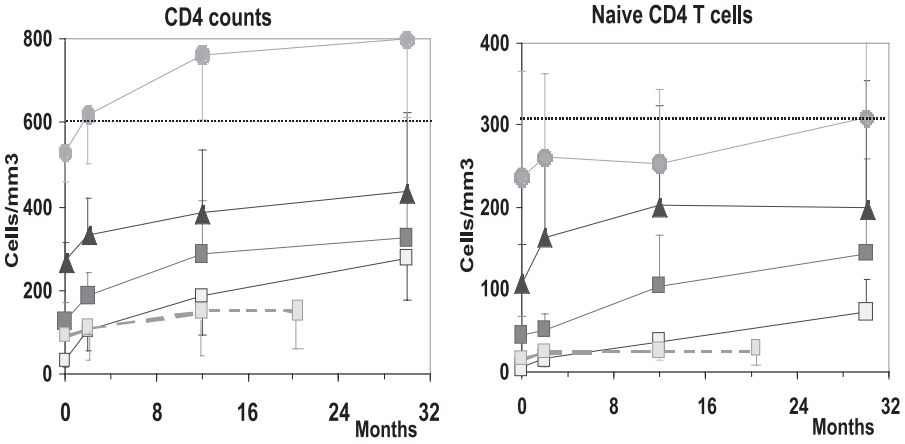

Figure 20.2. Profiles of total and naive CD4 cell reconstitution during HAART as a function of the disease stage. The slopes of reconstitution are roughly parallel in early and late disease stages. In contrast, naive CD4 cells show a rapid increase at early stages but a slow and delayed increase at the AIDS stage. In some rare occasions (< 5%), lack of total CD4 and naive CD4 cell recovery contrasts with the efficient suppression of virus replication. Data are calculated from 45 patients receiving HAART, none of them being drug-experienced, with viral loads successfully controlled (<200 copies/ml) (Autran et al., 1997; Carcelain et al., 1999; Li et al., 1998; Renaud et al., 1999; unpublished data).

results were confirmed by various authors (Lederman et al., 1998; Pakker et al., 1998), thus strengthening the hypothesis of a preserved capacity to regenerate naive T cells during HIV infection in adults. Computed tomography confirmed the preservation of a thymic tissue in HIV-infected adults and its progressive increase in parallel to naive CD4 T-cell amplification during treatment (McCune et al., 1998). Direct evidence for a thymic participation and T-cell regeneration during antiretroviral therapy came from the molecular detection of markers of thymic origin in these subsets of naive T cells (Douek et al., 1998; Poulin et al., 1999; Zhang et al., 1999a). Indeed, the DNA episomal circles produced within the thymus during rearrangements of the T cell receptor (TCR) segments do not replicate with the cell genome. Their detection in naive CD4 T cells shows that those cells have not undergone multiple cycles of proliferation since leaving the thymus: high proportions of the TCR rearrangement excision circles, or TRECs, are found in the subset of phenotypically defined $CD45RA^{+}CD62L^{+}$CD4 T cells, although only one-tenth of the phenotypically defined naive CD4 T cells harbor TRECs (Douek et al., 1998). Thus, the relative proportions of TRECs within the CD4 cell subsets fits with the phenotypic definition of naive and memory T cells. Once a naive cell emigrates from the thymus, it does not proliferate until it encounters a specific antigen, and then converts into a memory T cell. Intervals between two cell cycles in a naive cell might reach 10 years (Sprent and Tough, 1994): Thus, naive T cells that have

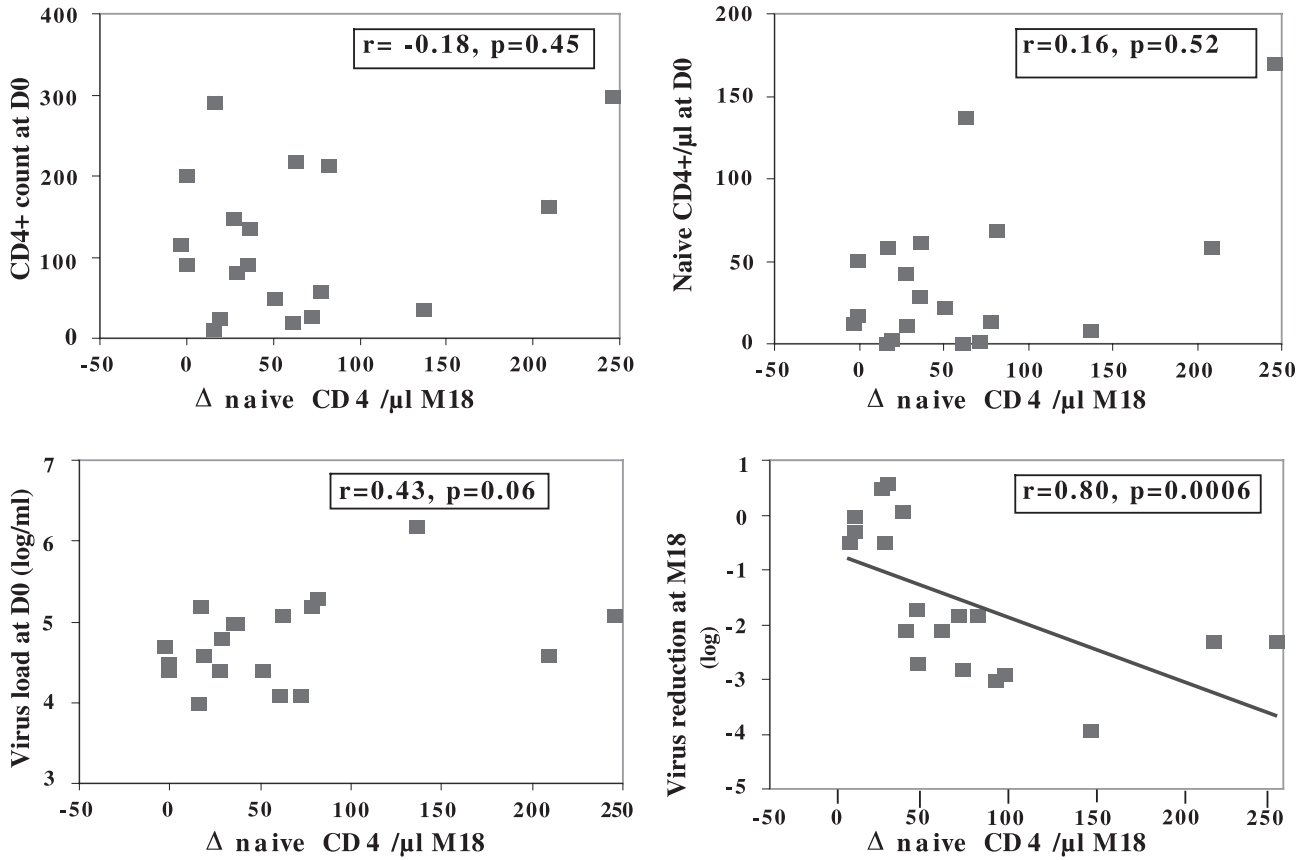

Figure 20.3. Long-term naive $CD4^+$ cell regeneration is independent of HIV disease at baseline but depends on long-term virus control. Calculations from naive CD4 T-cell analysis on blood samples (Li et al., 1997; Neumann et al., 1999; unpublished data).

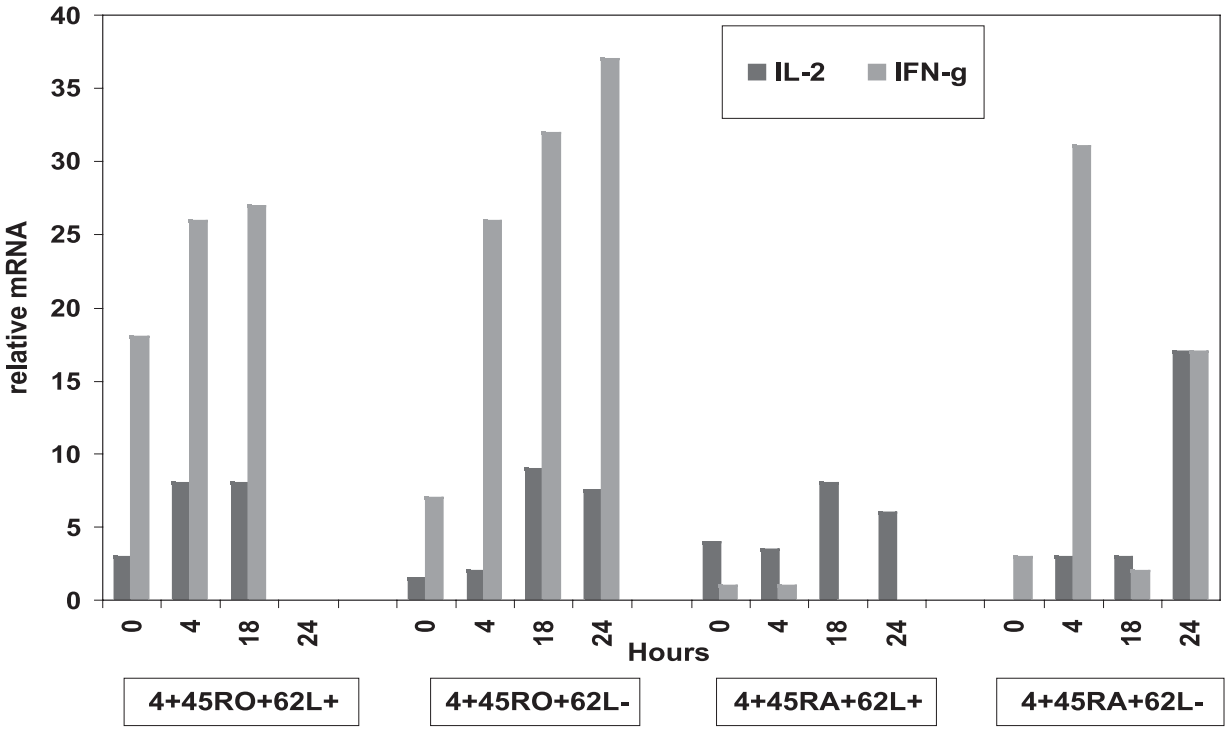

Figure 20.4. Phenotypic definition of naive and memory CD4 T cells and cytokine profiles. A flow cytometry sorting was performed on peripheral blood lymphocytes from a normal donor on the indicated subsets. A semi-quantitative reverse transcriptase polymerase chain reaction analysis was performed at various time points after stimulation using a combination of anti-CD3 and anti-CD28 antibodies.

not proliferated for 8–10 years might still contain their TREC copy and are defined as recent thymic emigrants (RTE). TREC proportions also decline with age and HIV infection (Douek et al., 1998; Zhang, 1999; Poulin et al., 1999). The rapid and sustained increase in TREC proportions observed during HAART in the majority of treated patients is correlated with the naive T-cell subset increases (Zhang, 1999; Poulin et al., 1999). This increased thymic production allows the repertoire of CD4 T cells to rediversify during HAART as shown by Gorochov and co-workers (Gorochov et al., 1998), thus helping to rediversify the host defenses. The hypothesis of an improved thymic function with HAART has, however, recently been challenged. Indeed, because the TREC proportions provide a good estimate of the peripheral T-cell turnover, a simple reduction in the intense immune activation observed during the natural course of the disease might result in a lower loss by conversion to memory T cells (Hazenberg et al., 2000). Altogether, however, strong evidence shows that thymic function was not definitively altered in the course of the disease and can help to refill the reservoir of naive T cells during HAART.

CORRECTION OF ABNORMAL ACTIVATION OF THE IMMUNE SYSTEM AND IMMUNE RECONSTITUTION

One of our first observations was a rapid and remarkable reduction in the cell surface expression of various T-cell activation markers such as human leukocyte antigen (HLA)-DR, CD38, or CD25, the interleukin (IL)-2 receptor, both on CD4 (Fig. 20.5) and on CD8 T cells after 2 months of virus reduction (Autran et al., 1997). The proportions of activated T cells significantly decreased in parallel to the reduction of the plasma virus load. Other groups reported a similar decrease in soluble markers of immune activation such as the inflammatory cytokines tumor necrosis factor (TNF)-α and IL-6 (Andersson et al., 1998; Lederman et al., 1998). In addition, a reduction in the Fas/Fas-ligand cell surface expression was also observed (Sloand et al., 1999). Both phenomena, reduced Fas expression and TNF-α levels, certainly help reduce the abnormal rate of cell death observed in HIV infection (Weichold et al., 1999) and restore normal numbers in the T-cell compartments. They also have important impacts on other immune cells such as B cells or polymorphonuclear cells.

These changes occur very early after virus begins to be controlled, both in the peripheral blood and in the lymphoid tissues (Anderson et al., 1998; Bucy et al., 1999; Sachsenberg et al., 1998; Tenner-Racz et al., 1998). Reduced immune activation is also associated with decreased cell surface expression of various adhesion molecules (Bucy et al., 1999), which certainly contributes to allowing cell recirculation. The same observations are made at any stage of the disease, in primary infection (Carcelain et al., 1999) as well as in chronic HIV infection.

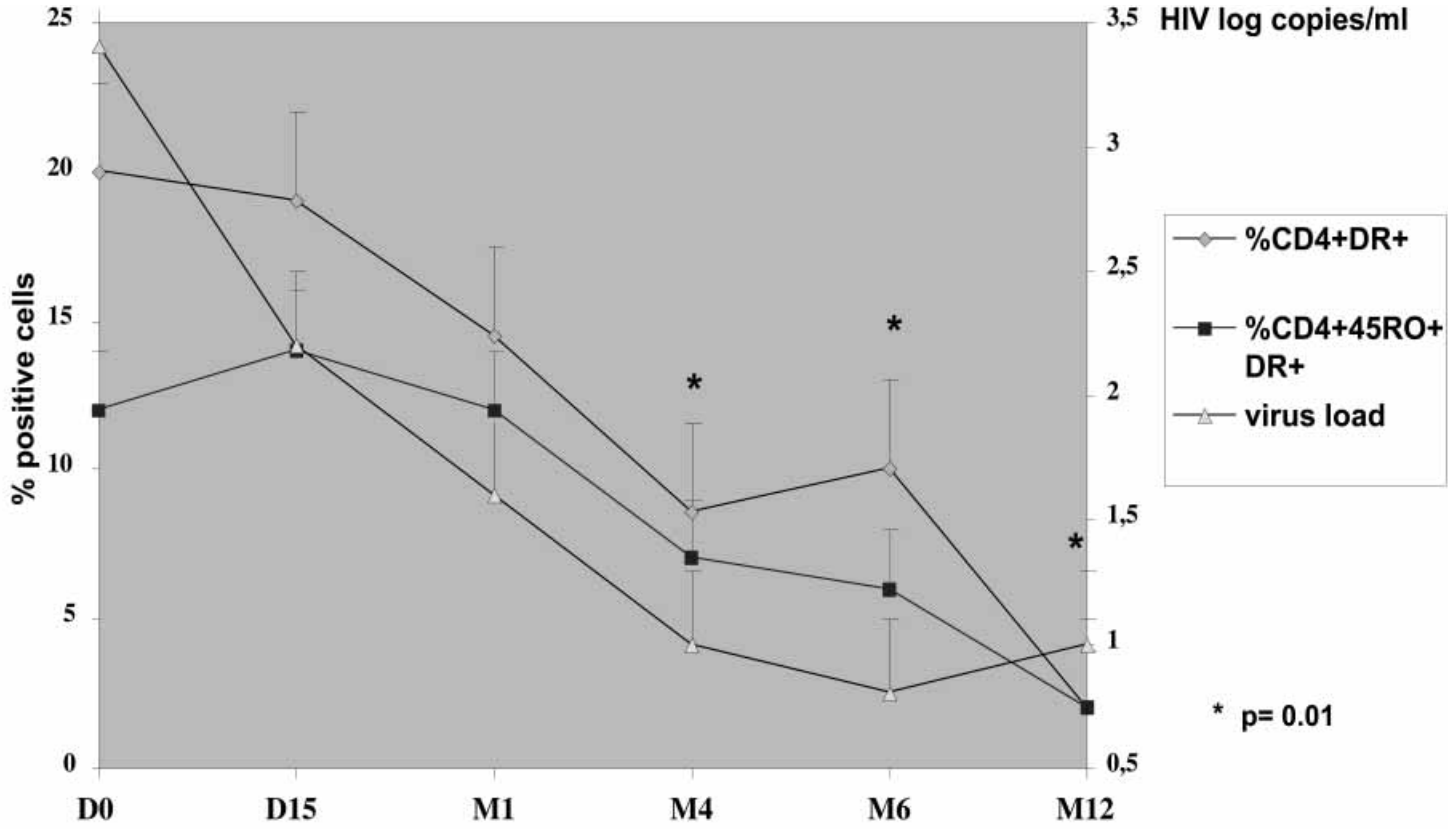

Figure 20.5. Treatment induces a parallel decrease in viral load and CD4 T-cell activation. Data from eight patients naive of antiretroviral drugs, initiating HAART with a median 150/mm^3 CD4 count (calculated from Autran et al., 1997).

Cell surface molecules related to T-cell activation function also progressively normalize. The CD28 coactivation molecule, down-modulation of which during the course of the disease contributes to cell anergy and sensitivity to apoptosis, reaches a full expression on 99% of CD4 cells during treatment of both primary and chronic HIV infection (Autran et al., 1997; Carcelain et al., 1999). In addition, the subset of CD4 cells that lacks the CD7 molecule and mediates a Th0/Th2 function slowly decreases and reaches normal values below 10% of the CD4 compartment after a year of successful therapy (unpublished observations).

Altogether, reduction in abnormal immune activation might be one of the key phenomena that allows, or at least contributes to, quantitative and functional immune reconstitution. Indeed, as discussed above, reducing the abnormal immune activation and losses in the memory T-cell compartment prolongs their survival, reduces the consumption of naive T cells, and helps restore their numbers (Hazenberg et al., 2000a, 2000b) while facilitating recirculation of the CD4 T-cell subset. Thus, antiretroviral drugs, by blocking virus production, reduce the virus-driven immune activation and play a potent anti-inflammatory role in the immune system. Overall, such anti-inflammatory effects might be even as important as thymic production for immune recovery.

RECONSTITUTION OF HOST DEFENSES DURING HAART: SUCCESSES AND LIMITATIONS

One of the major questions raised by physicians and patients was whether restoring CD4 cell numbers and homeostasis would restore the defenses that had been lost with AIDS. The phenotypic improvements reported above strongly suggested that the functional defects in memory T cells would also be corrected.

Indeed, T-cell function studies rapidly showed that CD4 T-cell responses against cytomegalovirus (CMV) and mycobacterium tuberculosis were shown to be restored after 3 months of HAART in AIDS patients (Autran et al., 1997; Lederman et al., 1998; Li et al., 1998; Pontesilli et al., 1999; Rinaldo et al., 1999; Valdez et al., 2000). Such reconstitution appears concurrently with memory CD4 cell increase and normalization of the inflammatory syndrome and requires a solid and durable control of HIV replication (Li et al., 1998; Wendland et al., 1999). Blocking HIV replication for only 3–6 months was not enough to restore a CMV or a tuberculin-specific immunity (Li et al., 1998). The classical T-cell proliferation assays are, however, poorly quantitative and could not assess increases in antigen-specific CD4 T-cell numbers. New flow cytometry analyses of the intracellular production of cytokines upon antigen stimulation helped demonstrate a quantitative restoration of CMV-specific CD4 T cells (Komanduri et al., 1998). These new methods clearly demonstrated an expansion in the frequencies of CMV-specific CD4 T cells that strongly correlated with the improved CMV disease status after introduction of HAART.

The clinical benefits of this restoration of immune defenses against opportunistic pathogens was rapidly confirmed by anecdotal observations of control of opportunistic infections (Li et al., 1999) and by epidemiological surveys (Buchbinder et al., 1999; Hogg et al., 1997; Jones et al., 1999; Pallela et al., 1998). Indeed, the incidence of opportunistic infections in treated patients recovering CD4 cell numbers $>200/mm^3$ was similar to the incidence observed in untreated patients $<200/mm^3$ (Grabar et al., 2000; Miller et al., 1999). Some controversies still persist over the delay of reconstitution depending on the age of patients (Chougnet et al., 1998) or the stage of the disease at which treatment is initiated (Clerici, et al., 2000).

Some early reports mentioned, however, recurrences of CMV retinitis after introduction of HAART (Gilquin et al., 1997; Jacobson et al., 1997). These rare observations can be easily explained by the delay of 3 months that is usually observed between introduction of HAART and reappearance of significant immune responses to pathogens. Indeed, initiating treatment at very low CD4 counts leaves patients with a prolonged risk of opportunistic infections that can still occur in the lag time before they reach sufficient immune reconstitution (Miller et al., 1999). Cohort studies demonstrated very clearly that reconstituting CD4 T cells $>200/mm^3$ restores protection against opportunistic pathogens and allows withdrawal of primary or secondary prophylaxis against CMV (Jouan et al., 1999; Tural et al., 1998) and *Pneumocystis carinii* (Furrer et al., 1999; Schneider et al., 1999; Weverling et al., 1999).

These successes have some limits inasmuch as no restoration of a CD4 cell reactivity against HIV itself was observed in chronically infected patients (Autran et al., 1997; Lederman et al., 1998; Plana et al., 1998; Pontesilli et al., 1999; Rinaldo et al., 1999). The CD4 helper cells specific for HIV were restored only when HAART was initiated at the time of primary infection (Al-Harthi et al., 2000; Rosenberg et al., 1997, 2000). We checked that those cells were not sequestered in the tissues (T.S. Li, unpublished). The regeneration of new naive CD4 cells does not help restore such activity even after 2 or 3 years of fully suppressive treatments. However, the HIV-specific T-helper cells were not definitively deleted during chronic infection but persisted at very low frequencies as shown by flow cytometry studies quantifying the frequencies of HIV-p24-specific CD4 T cells producing interferon (IFN)-γ (Pitcher et al., 1999). This question of T-helper cell activity against HIV seems the most important, considering the central role played by those cells in the generation and maintenance of the effector immune defenses mediated by CTLs and antibodies (Kalams and Walker, 1998; Matloubian et al., 1994). One of the best hallmarks of this unrestored immunity against HIV is the rebound of uncontrolled virus replication observed when HAART is stopped (De Jong et al., 1997; Neuman et al., 1999). Again, only early treatment of primary infection led to some control of the virus once the treatment is stopped (Lisziewicz et al., 1999; Ortiz et al., 1999; Rosenberg et al., 2000).

Such a dissociation between a strong and protective immunity rapidly restored against opportunistic pathogens and a waning immunity against HIV

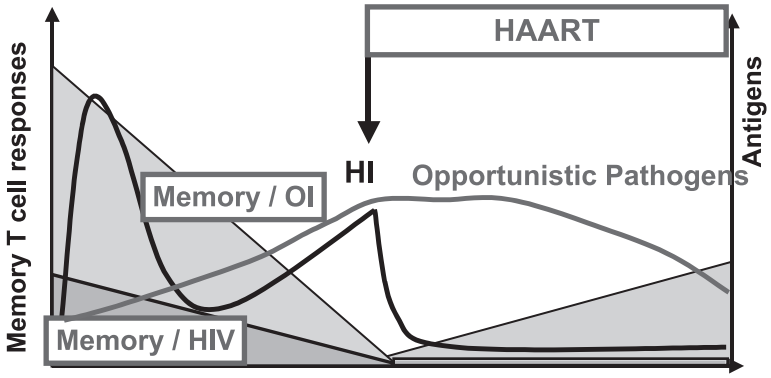

Figure 20.6. Memory CD4 T cell re-expansion with HAART requires re-exposure to antigens. The rapid viral load reduction allows an early reappearance of CD4 cell responses to opportunistic antigens that contrasts with the lack of restoration of CD4 T-cell responses to HIV antigens. This phenomenon can be explained by the distinct exposure of these various T cells to their respective pathogens. HAART directly reduces production of HIV antigens but does not directly affect production of opportunistic pathogens that are present in quantities sufficient to allow re-expansion of memory T cells.

might be explained by the differences in the exposure to appropriate antigenic stimuli (Fig. 20.6). Significant virus production seems to be required for appropriate stimulation, proliferation, and re-expansion of antigen-specific CD4 T cells. Because the amounts of CMV or *Candida* antigens are not immediately affected by HAART, the resurging memory CD4 T cells specific for those pathogens are rapidly restimulated and can re-expand. In contrast, production of HIV antigens rapidly disappears with HAART and the specific CD4 cells cannot be appropriately restimulated or expanded (Fig. 20.6). Similarly, a decline of HIV-specific CD8 T cells (Dalod et al., 1998; Gray et al., 1999; Kalams 99; Mollet et al., 2000; Ogg et al., 1999) and antibody titers (Morris et al., 1998) was reported to parallel virus reduction. The hypothesis of a bolus of HIV antigen too low to allow memory T-cell expansion might contrast with the findings of a persisting reservoir of virus in tissues (Chun et al., 1997; Finzi et al., 1997; Zhang et al., 1999b). This reservoir is, however, replicating at a rate too low to stimulate appropriately T cells. The treated patients are thus left with a strong and protective immunity against any pathogen except HIV itself.

Such partial immune reconstitution might also reflect some defect in the ability of the antigen-presenting cell network to efficiently prime new naive CD4 and CD8 T cells. We demonstrated, indeed, that a defect in the frequencies of peripheral blood dendritic cells expressing the CD11c molecule and producing IL-12, which was not restored during treatment (Grassi et al., 1999).

Memory against HIV persists, however, even at very low levels, and is reinducible. Peaks of HIV-specific CD4 cell numbers have been registered during short periods of virus replication when treatment fails or is interrupted (Carce-

lain et al., 2001). These observations led to the concept of structured therapeutic interruptions (STI), which might re-increase HIV-specific immunity by transiently but recurrently re-exposing the patient to HIV virus particles during treatment windows. This strategy might have some benefit when applied after treatment of primary infection (Lisziewicz et al., 1999; Ortiz et al., 1999; Rosenberg et al., 2000). We reported, however, that re-expansions of HIV-specific CD4 T cells were usually only transient in chronically infected patients (Carcelain et al., 2001).

Another safer strategy is currently proposed to restore HIV-specific immunity and to limit drug toxicity in treated patients by re-immunizing patients against HIV with candidate vaccines (Autran and Carcelain, 2000). Inactivated HIV particles and recombinant viral vectors, such as canary pox (Goh et al., 2000) or DNA immunizations (Calarota et al., 1999), are currently being tested for their ability to restore CD4 T-cell responses to HIV proteins in treated patients (Valentine). Encouraging preliminary results also suggest that cytokine therapy such as IL-2 might help accelerate immune reconstitution (Chun et al., 1999; Cooper and Emery, 1999; Hengge et al., 1998; Imama et al., 1999; Katlama et al., 2000; Kovacs et al., 1996). Immune-based strategies aimed at restoring immunity against HIV might also incorporate other adjuvant cytokines (Landay et al., 1996; McFarland et al., 1998) that enhance the functions of antigen-presenting cells and their capacity to generate strong and protective CD4 Th1 and CD8 cell responses against HIV.

CONCLUSION

In conclusion, new regimens of antiretroviral therapy have successfully restored a normal or subnormal CD4 compartment and protection against opportunistic pathogens. The major condition required for immune reconstitution is an efficient and durable inhibition of virus replication. These successes demonstrate that HIV does not definitively alter the lymphoid tissues or the immune defenses, even after years of infection and severe immune suppression. Despite the many controversies about the fine mechanisms of such recovery (Miedema, 2000) or about whether the immune system can perfectly recover, many factors are at work (McCune). Three mechanisms certainly allow CD4 recovery: 1) redistribution of memory CD4 T cells from tissues where they had been previously sequestered; 2) regeneration of naive T cells from thymic origin; and 3) reduction of the inflammatory syndrome. Initiating HAART in late or in early disease might differ, however, by the time required to reach normalized immune functions and cell counts and by the lack of amplification of HIV-specific CD4 T cells in late disease. It is obvious that only long-lasting control of HIV allows immune restoration (Saag, 1999). Lack of reconstitution of a solid immunity against HIV itself is currently increasing the need for additive therapeutic strategies based upon immune interventions.

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New Perspectives in Antiretroviral Therapy of HIV Infection

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INTRODUCTION

The introduction, in 1995–1996, of the concept of maximal suppression of human immunodeficiency virus (HIV) replication together with the availability of potent antiretroviral combinations produced a dramatic impact on the natural history of HIV disease, with unprecedented changes in disease progression and mortality in the United States and in western countries (Palella et al., 1998; Sendi et al., 1999). In this part of the world, despite the unsuccessful attempts to eradicate HIV infection from an infected individual (Finzi et al., 1999), at least a first important goal seems to have been obtained: possibility of turning HIV-1 infection from an inevitably fatal disease into a chronic condition, potentially manageable over the course of decades.

However, despite impressive progress, much work remains to be done to reach this target. Even with the most potent regimens available today, a significant proportion of patients fail to have a complete virological response after their first-line treatment. This population is at high risk to become less responsive to subsequent antiretroviral regimens because of development of HIV resistance to the antiretroviral drugs in their regimen. Moreover, incomplete response rate and limited durability of the response are not the only shortcomings of the current regimens. Other problems include complexity of pharmacokinetic interactions with other drugs, burden of pills, and constraints in timing of adminis-

tration that dramatically impact adherence to the regimens. All available drugs also have important short- and long-term toxicities, and broad cross-resistance can emerge among existing drugs of the same class.

CONSIDERATIONS ON WHEN TO START ANTIRETROVIRAL THERAPY

Controversies continue over the best time to initiate antiretroviral therapy (Carpenter et al., 2000; US-DHHS, 2000). Ideally, to prevent progressive or irreversible immune damage, treatment should be initiated early in the course of the disease. Early initiation of antiretroviral treatment seems justified for several reasons: HIV infection almost invariably causes progressive immune damage; disruption of the immune system and the building of viral reservoirs are early events; and the natural history of untreated HIV infection includes selection for more diverse and more virulent strains of HIV.

Early start, however, must be balanced against the drawbacks of long-term therapy. Evidence is now present that the risk of disease progression is low until substantial CD4 cell loss has occurred, and that immune recovery is impressive even in delayed therapy (Autran et al., 1999; Kaufman et al., 2000). Early initiation of treatment may expose patients to unnecessary medication-related risks, which range from adherence problems and impact on quality of life to the potential for early development of resistance to antiretroviral agents and the possibility of serious metabolic complications.

Moreover, the final target of therapy is not viral load per se, but preservation of the overall health of the patient, taking care of the quality in addition to the quantity of life. It is well known that long-term adverse effects of therapy are sufficiently serious, and many patients achieve only incomplete or transient control of viral replication, resulting in selection for resistant strains of HIV. Given all these reasons, it is now considered inappropriate to initiate therapy, in the asymptomatic individual, at a time when the CD4 count indicates no appreciable risk for serious opportunistic infections. This has translated, in the past 2 years, into an increasing tendency to defer initiation of therapy until immune deficiency becomes relevant and the risk of disease progression becomes significant.

The following criteria may guide selection of patients for initiation of therapy:

- All patients with symptomatic HIV infection, regardless of $CD4^+$ count and viral load levels and all patients with $CD4^+$ counts $<350/mm^3$ should start antiretroviral therapy.
- Treatment could also be considered for patients with a high viral load (i.e., $>50{,}000$–$100{,}000$ copies/ml) regardless of the absolute number of $CD4^+$ lymphocytes, and for persons with $CD4^+$ cell counts between 350 and $500/mm^3$.

- Treatment of asymptomatic patients with $CD4^+$ cell counts >500 mm^3 should be deferred as long as the probability of significant immune system damage and of clinical progression of HIV infection remains low.

It should be remembered that $CD4^+$ and HIV-1 RNA are good but incomplete surrogate markers for both natural history and treatment effect, that thresholds are arbitrary, that the disease process is a biologic continuum, and that treatment decisions should be individualized according to both baseline and the trajectory of the available immunological and virological markers (Chaisson et al., 2000).

Initiation of therapy must be fully discussed with the HIV-infected person, who should begin a regimen only if he or she is committed to complete adherence and fully understands the advantages and the potential drawbacks and burden of antiretroviral therapy.

Finally, it has to be considered that current debate surrounding initiation of therapy underscores inadequacies of current agents. When simple, potent, durable, nontoxic regimens become available, the pendulum may swing again toward a more aggressive approach.

GOAL OF ANTIRETROVIRAL THERAPY AND AVAILABLE OPTIONS FOR THERAPY INITIATION

Data from several studies indicate that the nadir of the virologic response and the rapidity with which virus replication is suppressed are strongly predictive of the likelihood of achieving a durable virologic response, suggesting that little or no virus evolution occurs in the residual virus population when plasma HIV-1 RNA is persistently suppressed to below the limits of quantification of ultrasensitive assays for viral load. In particular, no evidence was found for the emergence of drug resistance mutations when viral sequences recovered from lymph nodes or peripheral blood lymphocytes of these subjects were analyzed.

As previously pointed out, physicians and persons living with HIV/acquired immunodeficiency syndrome (AIDS) need, therefore, to weigh the risks and benefits of starting antiretroviral therapy and make individualized decisions about when to initiate therapy. However, once the decision to start has been made, the ultimate goal of treatment should be the maximal suppression of HIV replication, because the major short-term risk of any continuing viral replication in the presence of antiretroviral therapy is the emergence of drug resistance.

To achieve this goal, particularly in persons being treated for the first time, several strategies are now available.

Regimens are usually composed of three or four drugs and may include (on a two nucleoside reverse transcriptase inhibitors [NRTI] backbone), one (or two) protease inhibitors (PI), or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Regimens including three NRTIs are also increasingly being used.

Investigational regimens include combinations such as one to two protease inhibitors plus NNRTI plus one to two NRTIs, and NRTI-sparing combinations such as one protease inhibitor (plus low-dose ritonavir) plus one NNRTI.

With short-term clinical trials indicating comparable potency and efficacy of PI-, NNRTI-, and all-NRTI-containing regimens, the attention should be rather focused on their differences with regard to long-term outcome, toxicity, constraints on future regimens, and the effect on viral reservoirs in non-lymphoid compartments. The answers to these questions and the ratio of the advantages to disadvantages of these different treatment strategies will only come from the results of well-controlled strategic clinical trials, such as ACTG 384, currently conducted by the Adult AIDS Clinical Trials Group (ACTG) of the National Institutes of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH), and the European INITIO study. Both of these large studies are aimed at investigating the effectiveness not just of single regimens, but rather of different therapeutic pathways, including a first-, second-, and eventually third-line regimen.

The choice of specific backbone NRTIs is based on convenience, resistance/toxicity profile, and patient preference. Some widely used NRTI combinations (not in preferred order) are zidovudine plus didanosine, zalcitabine, or lamivudine; and stavudine plus didanosine or lamivudine. AZT and d4T should not be administered together because of a negative interaction at the cellular level.

PI-containing regimens (two NRTIs plus one PI) are very often used as first choice for initiating antiretroviral therapy, and there are sufficient data on the long-term effectiveness of these regimens. PI regimens have proven potency and are effective in patients at all levels of plasma viral load. However, there are important disadvantages that limit their acceptability: a) the complexity of the regimens makes adherence difficult; b) cross-resistance between different PIs may limit future use should initial therapy fail (and some clinicians may wish to spare PI for second-line regimens); and c) there is growing concern over the long-term toxicity of PIs, particularly the fat redistribution and the metabolic abnormalities whose effect on long-term cardiovascular morbidity and mortality remains uncertain.

Combinations of two PIs are increasingly being used instead of a single PI because they have pharmacokinetic advantages (i.e., increase of drug plasma levels and possibility to use a twice-a-day schedule of administration) and therefore increase the PI regimen's potency while greatly improving adherence to therapy. Addition of a low dose of ritonavir (100 or 200 mg twice a day) to saquinavir, indinavir, or amprenavir improves the pharmacokinetic profile, may reduce pill burden, lower the dose frequency, lower cost, and obviate the need for administration of PIs on an empty stomach. A co-formulation of lopinavir plus ritonavir, based on an extremely potent interaction, has been recently introduced. The long-term benefit and toxicity of dual PI combinations remains to be fully characterized.

Combinations between NNRTIs and NRTIs have recently gained popularity as "PI-sparing" regimens. There is convincing evidence from controlled clinical trials that, in treatment-naive patients, NNRTI regimens offer a suit-

able alternative to PI-containing combinations in terms of potency. Besides the advantage of deferring the introduction of PIs, NNRTI-containing regimens may also allow for a lower pill burden and therefore improved adherence. The main disadvantage of NNRTIs is the ease and rapidity with which resistance develops to the individual drugs in this class if they are used in the context of a regimen that is not maximally suppressive and the very strong likelihood that cross-resistance within this class of drugs will follow.

The use of three NRTIs to ''spare'' both PIs and NNRTIs has recently been proposed. Most data refer to the combination of abacavir, zidovudine, and lamivudine, which has shown durable antiviral activity (after 48 wks of treatment), equivalent to that of a ''standard'' two NRTI plus one PI regimen (e.g., zidovudine/lamivudine/indinavir), in treatment-naive patients. This combination, however, could be less effective in patients with high baseline plasma viral loads. The main attraction of a three NRTI regimen is deferral of the use of PIs, while also sparing the NNRTI and placing only a single class of antiretroviral drugs ''at risk'' for the development of resistance. Once again, the long-term efficacy and toxicity of multinucleoside regimens remains unknown and there is concern over the potential possibility of selecting for multinucleoside-resistant variants of HIV and over the possibility of a cumulative nucleoside toxicity.

In planning first-line therapy, the following considerations may apply: for patients with viral loads <100,000 copies/ml, PI, NNRTI, or triple NRTI (w/ABC) based regimens are probably comparable options in terms of efficacy. For patients with high viral loads, more data are needed regarding efficacy of triple NRTI abacavir (Ziagen, ABC) (w/ABC) regimens.

A major cause for concern is the evidence from several clinical trials that 20–40% only of previously untreated patients achieve complete virologic suppression (defined as plasma HIV-1 RNA below the limits of detection), even with the currently available potent regimens. Evidence from several laboratories making use of sensitive molecular assays suggests persistent virus replication in lymphoid tissues of at least some of these patients. Such persistent replication may be responsible for the occasional ''blips'' in plasma HIV-1 RNA that are observed in some patients. Intermittent nonadherence, interindividual variation in pharmacokinetics, drug-to-drug interactions, and inadequate potency of current regimens could concur in determining persistent virus replication. Given the high rate of HIV-1 replication, the concern is that any residual turnover could lead to rapid repopulation of the HIV reservoirs. Therefore, the use of antiretroviral combinations that produce high activity on viral reservoirs (anatomical and cellular) is also being explored as a tool to improve overall efficacy.

COMPLICATIONS OF ANTIRETROVIRAL THERAPY

Important factors that negatively impact long-term outcomes are complications of therapy and its recently emerged untoward effects, specifically metabolic/fat abnormalities and mitochondrial toxicity (which has several manifestations

including lactic acidosis, peripheral neuropathy, lipodystrophy, pancreatitis, myopathy, and anemia). All drugs in the NRTI class may cause potentially fatal lactic acidosis with hepatic steatosis. A potential explanation is mitochondrial toxicity, generally attributed to inhibition of mitochondrial DNA polymerase by nucleoside analogs. Dyslipidemia and changes in body fat distribution (often termed lipodystrophy), although more common with PI, have also been observed in patients treated exclusively with NRTIs and could also be associated with the prolonged use of nRTI regimens.

All drugs in the NNRTI class (nevirapine, delavirdine, and, to some extent, efavirenz) may cause rash and liver toxicity. Most cases of rash are mild or moderate. However, cases of Stevens-Johnson syndrome have been described. Elevations of aminotransferases, and cases of fatal hepatitis have been reported, particularly with nevirapine. Efavirenz may cause transient central nervous system disturbances that manifest as a complex of sleep and mood disturbances.

Metabolic dysfunctions with dyslipidemia and/or changes in body fat distribution are more commonly associated with use of PI-containing regimens (Carr, 1999). Symptoms are variable and may include peripheral fat loss and central fat accumulation, often associated with hypercholesterolemia, hypertriglyceridemia, insulin resistance, and, rarely, hyperglycemia. Overall cumulative incidence may be 30% to 60% after 1 to 2 years of therapy and increases with the duration of therapy (Maus, 2000).

In most cases, we have to admit that the pathophysiology of the adverse effects of antiretroviral drugs is not fully understood and that universally effective therapeutic interventions to counteract them are lacking or not established. Delaying the initiation of antiretroviral therapy seems today the only option. However, alternatives may arise in the future, particularly if clinical predictors, and genetic or pharmacological correlates are identified, and toxicity prophylaxis and treatment strategies are developed. Among these, intermittent therapy is being explored as a promising approach, together with substituting protease inhibitor with other classes of compounds to minimise PI-related toxicities and ease adherence. Both abacavir and nevirapine can be substituted for a PI, and data was recently presented to show that efavirenz could also be a useful substitutes. It should be emphasised that before recommending substitution, such strategies are only suitable for patients who have suppressed viral load and have never had virological failure on the current or prior regime.

TREATMENT FAILURE AND HIV RESISTANCE TO ANTIRETROVIRAL DRUGS

Treatment Failure

The reasons for treatment failure in HIV-1 infection are multifactorial, including evolution of drug resistance, pharmacokinetic and metabolic factors, in-

adequate drug potency, nonadherence, and still unknown factors. Indeed, incomplete adherence is the most common cause of virologic failure (the level of adherence needed to maintain an adequate response is higher than that described for any other disease) (Haubrich et al., 1999).

Adherence should be assessed and reinforced as a routine part of management. However, many ways of improving adherence are being investigated, but they remain largely empiric (Paterson et al., 2000).

Any discussions on salvage strategies requires a previous definition of parameters of treatment failure. For the adherent patient on an initial treatment regimen, confirmed detectable plasma HIV-1 RNA should be considered evidence of treatment failure. Continued treatment with the same regimen in this situation will eventually lead to development of high-level drug resistance, and diminishes the likelihood that salvage regimens will be successful. Thus, for the patient with clear treatment options, early switching could maximize the chances for therapeutic success of the next treatment regimen and preserve future options.

The situation differs for patients who are highly treatment experienced and for whom fewer options remain. In such cases, a more conservative approach may be warranted. Usually, virologic escape is followed by immunologic deterioration and eventually clinical evolution. However, the time lag between HIV RNA rebound and clinical failure varies from patient to patient, and it has become clear that $CD4^+$ cell count may remain high even in the presence of a clear rebound in HIV RNA. Before making any decisions about changes in antiretroviral treatment, it is important to determine why the current regimen is failing to avoid choosing an inappropriate remedy. In particular, factors such as drug resistance, inadequate drug exposure due to poor adherence, absorption and pharmacokinetics, and persistence of HIV in viral reservoirs all represent major causes of failure that should be deeply investigated before switching to a different regimen.

Unfortunately, due to the large degree of cross-resistance occurring within all antiretroviral drug classes, limited options actually exist, especially for patients experiencing their second or third failure. In this heavily pretreated population, both observational and prospective studies have shown quite disappointing results of any investigated salvage regimens.

New strategies in the management of treatment failure are being investigated, including structured treatment interruptions, new combinations (triple PI, dual NNRTI), exploiting drug ''hypersusceptibility'' (which has been described with zidovudine, abacavir, NNRTIs, amprenavir, and saquinavir), and reduced viral fitness. Indeed, the scientific basis for continuing activity of drugs in failing regimens (Deeks et al., 2001) is supported by data that suggested that drug-resistant viruses were less fit than wild-type ranging from a 3.3 to 36.1% relative loss of fitness. Interestingly, the less fit the mutant virus during therapy the more rapidly wild-type virus rebounds when a treatment interruption was started. Whether the loss of fitness could be exploited more in salvage patients is one area of study that should be intensified.

Role of Resistance Testing

In the next years, a widespread diffusion of genotypic resistance testing is expected. Though representing a major advantage for the management of HIV patients, the routine use of these tests in the clinical setting has yet to be validated. In general, whereas resistance is generally a good predictor of likely failure of a drug, susceptibility is no guarantee of success. It is becoming a common belief, therefore, that these assays have their major application in predicting which drugs not to use rather than which are likely to be successful. Their best current application is therefore the design of salvage regimens. Indeed, several retrospective and a few prospective studies have shown that a higher response rate is likely to occur when a salvage regimen is selected on the basis of genotype or phenotype testing results.

Indeed, the major advantage of resistance testing availability is that the results can also help sparing drugs when a new regimen has to be designed. Although the general principle of changing all drugs in the new combination keeps all its validity, in some cases, drug failure may not imply resistance to all agents in that combination and therapeutical options may be limited. In these settings, changing one drug only may restore sensitivity to the whole regimen.

However, there are several inherent limitations that should be kept in consideration when interpreting the results of both phenotypic and genotypic esistance testing, such as their ability to measure only the predominant viral species and the fact that some mutations conferring significant resistance to one drug may actually increase viral susceptibility to a second, unrelated agent (Hirsch et al., 2000).

Finally, transmission of drug-resistant virus at primary HIV infection and identification of drug-resistant virus in previously untreated patients is a growing phenomenon. Data from the United States show that the prevalence of high-level resistance to NNRTI and PIs has increased in the last year compared with 1996–1998. Ten- or greater fold resistance to NNRTI has increased from 1 to 7%, and for PIs from 2 to 6%. In Europe, drug resistance rates seems to have gone down in Switzerland, although clusters of primary HIV infection are occurring. In France, during 1999, the frequency of drug resistance was 6.5% for NNRTIs, 3.7% for NRTIs, and 2.8% for the PI. Continuing surveillance is required and many authors suggest that drug resistance testing should be performed in patients with primary HIV infection before initiating antiretroviral therapy.

NEW TREATMENT STRATEGIES

Currently, 15 antiretroviral agents are approved or available through expanded access programs, while there are at least 23 new drugs in clinical development that are expected to become available within the next 3 years. Several of them belong to the already well-known classes of reverse transcriptase inhibitors and protease inhibitors, and are characterized by higher potency and/or tolerability

and/or better pharmacokinetic profile compared with existing drugs. Furthermore, in some cases they show antiviral activity also against HIV strains that are no longer sensitive to previous antiretroviral agents.

A second group of new antiretrovirals consists of drugs acting on different viral targets. The most advanced drugs in development are virus-attachment inhibitors and fusion inhibitors that block de novo infection and cell-to-cell virus transmission. A further class of drugs includes the integrase inhibitors, which prevent the integration of viral DNA into the host DNA.

New drugs with improved tolerability and pharmacokinetic profile are eagerly awaited. However, the major advances in antiretroviral therapy are expected from the development of new treatment strategies.

Simplification of Therapy

Given the complexity of many current treatment regimens and the difficulties in treatment adherence experienced by many patients, the question of treatment simplification naturally arises from practical issues.

Unfortunately, the results so far available have been disappointing, at least for the studies that have been published and in which potent antiretroviral treatment with three or four drugs was started and continued for 3–6 months. Patients in whom plasma HIV-1 RNA was successfully suppressed to below the limit of detection were then randomized to continue the ''induction'' regimen or switch to a ''maintenance'' regimen consisting of fewer drugs. In all trials, the results were disappointing, patients randomized to maintenance regimens experiencing significantly higher rates of virologic failure as compared with those who remained on their initial therapy. However, several factors could account for the poor outcome of these studies: the duration of induction treatment, the virologic cutoff adopted for the switch to a simplified regimen, the low potency of both induction and maintenance regimens. While teaching us that potency must not be sacrified solely for the sake of improved adherence, the failure of the simplification strategies so far investigated should not discourage us from designing further trials, the key issue being design of ''simpler'' rather than ''weaker'' regimens (Montaner, 1999). A variation on the theme of induction maintenance may be starting with one proven, potent regimen, then switching to avoid or ameliorate toxicity and/or adherence. Although numbers of drugs in the regimen may not change, dosing regimen and/or pill burden may be better.

Examples of this strategy include switching from PI (plus or minus ritonavir) plus dual NRTI combination to NNRTI/dual NRTI or triple NRTI and from PI or NNRTI/dual NRTI combinations to a PI/NNRTI.

Treatment Intensification

Despite major success, the toxicity and complexity of the currently available antiretroviral drug regimens represent a major barrier to adherence and repre-

sent the major cause of virological failure. Furthermore, in spite of the apparent large number of drugs and the high number of theoretical combinations, cross reactivity among drugs of the same class means that therapeutic options remain limited. More potent drugs that are safer and easier to administer, with better pharmacological properties and with activity against drug-resistant viruses are therefore needed. In the meantime, all efforts should be directed toward prolonging the effect of the initial antiretroviral treatment. Indeed, many controlled studies have proven that the nadir of initial response correlates well with the durability of response and that achieving an early sustained response (by week 4 and/or 8 of therapy) is predictive of subsequent virologic suppression (Raboud et al., 1998). Plasma HIV-1 RNA levels should decrease rapidly after therapy is initiated (a minimum of a 1.5–2.0 log decline should occur over the first 4 weeks of therapy) and failure to achieve the target level of <50 copies/ml by 16–24 weeks (although in persons with higher plasma HIV RNA levels at baseline, e.g., $>100{,}000$ copies/ml, maximal viral suppression may take longer) should raise concern and prompt review of drug adherence, drug absorption, or the presence of drug-resistant virus. Intensification strategy is actually investigated in two different situations: failure to establish initial control of virus replication and "early" treatment failure after initial complete virological response. In persons with low but detectable HIV RNA following 12–16 weeks of potent therapy (but without identified resistance to drugs in current regimen), or with early HIV-RNA rebound after full suppression, the addition of a new drug could be an alternative to a complete change, after other causes of the treatment failure (particularly incomplete adherence) are ruled out.

Advantages of intensification strategies may include the fact that the lack of sufficient potency of a regimen does not necessarily imply drug resistance and that this strategy may allow preservation of drugs that remain potentially useful. Moreover, it could avoid initial use of additional drugs in persons who may not need them.

However, disadvantages of intensification strategies include the fact that inadequate virologic response could be due to the presence of drug-resistant variants. In this case, because there is a thin line between intensification and incremental therapy, further drug resistance may be promoted. Moreover, intensification could aggravate problems with adherence in the poorly adherent patient and could complicate problems with drug interactions.

Structured Treatment Interruptions

Two different approaches are guiding the ongoing efforts to define the role of structured (or strategic) treatment interruptions (STI) in current antiretroviral strategies.

The original targets for STI strategy were persons having experienced multiple failures and with no residual therapeutic options. The principle behind STI was a possible reversion of the mutant virus to the wild type, with a potential

restoration of drug susceptibility allowing for antiretroviral drugs' recycling. Unfortunately, the reversion to the wild type is frequently seen in plasma virus, but not in cell-associated virus, whereas a marked increase in viral fitness occurs during therapy interruption Finally, the heavy decrease in CD4 cell count and the long period of time required for their recovery, possibly associated with a high incidence of new clinical events, cast serious doubts on the advantage of performing STI in persons with advanced HIV disease.

STI in fully suppressed persons follows a quite different philosophy. Though eradication of HIV does not seem to be a feasible goal even with the most potent and timely interventions in use today, evidence that a specific and effective cellular immune response to HIV occurs in infected subjects has led to the exploration of alternative approaches to therapy that would aim at enhancing the host immune response. The hope is that short treatment interruptions may be able to augment the length and strength of host immune responses to HIV and increase immunologic control of the infection (Haslett et al., 2000; Lori et al., 2000; Rosemberg et al., 2000). Therapeutical holidays may also help in preventing or reducing side effects. Results from a few uncontrolled studies are available and the majority of persons seem to experience a rapid rebound of plasma HIV RNA during treatment interruptions as well as a rapid decline in CD4 counts, so that the possibility of boosting the HIV-specific immune responses through this approach therefore remains controversial and should be clarified by controlled studies (Ruiz et al., 2000). However, the following conclusions can be drawn from the limited experience so far: STI appear to be safe, as shown by the prompt reduction in HIV RNA and increase in CD4 that follow the reinstitution of treatment and by the conserved profile of drug susceptibility of the rebound virus. In a variable proportion of individuals, the increase in HIV-specific CD4 lymphoproliferative activity and some favorable effects on breadth and magnitude of cytoxic T lymphocyle response has been observed. In a few cases, a reduced virological setpoint has been seen after repeated STI, although we do not know whether the observed reduction in virological setpoint is really due to STI or whether it is just a casual event (such as normally seen in wide cohorts of HIV^+ individuals) and for how long will specific immune responses induced by STI persist.

Another possibility stands behind the need to carefully test, in the appropriately controlled trial setting, the strategy of structured treatment interruptions. Because it became evident that HIV/AIDS care in the third millenium implies long-term management of a chronic disease, with limited pharmacological options, the logical consequence is the need for durable therapeutic strategies, keeping in mind that adherence and quality of life issues strongly limit the long-term use of currently available antiretroviral regimens.

The current paradigm is that treatment of HIV must be continuous and for life. This is based on present knowledge of HIV dynamics under therapy. The perception is that cessation of treatment would lead to immediate reversion to pretreatment clinical status and be detrimental to the patient. However, ''un-

structured'' treatment interruptions very often occur in the real world because of drug toxicity, intercurrent illness, boredom with treatment, chaotic life style, and uncertainty over therapeutic effects. Many patients still wish to stop therapy temporarily (or permanently), and it is the unplanned treatment interruption (UTI) that currently dominates the field. Therefore, STI strategies may go quite beyond the issue of re-stimulating immune response for persons maximally suppressed, or to drive back wild virus in persons with multiple failures and multidrug-resistant virus: to ''give patient's a break,'' with the ultimate goal of prolonging the beneficial effects of antiretroviral treatment over the long term (and, on another note, the concept of STI may also have a unprecedented impact, if proven, in resource-poor settings).

However, many questions still need to be answered in controlled clinical trials before this strategy can be adopted: what are the long-term effects on viral load and CD4 cell count? Will the response to therapy reinstitution be reproducible over long time periods? What occurs in terms of genotypic/phenotypic resistance over time? Is STI strategy comparable, superior, or inferior to therapeutic vaccination? Will the response to STI be the same for persons having received PI or non-PI-containing regimens? What happens during STI in the different HIV compartments and reservoirs?

In conclusion, structured treatment interruptions, intermittent treatment, or pulsed therapy has distinct attractions for patients and healthcare providers, with potential for reduced toxicity, improved tolerance, greater adherence to dosing schedules, and lower cost. These first studies of interrupting HIV treatment seem to challenge the assumption that only harm would occur and to support the evidence that the virus is more forgiving than has been realized.

Immune Intervention

A remarkable amount of data has been recently produced, showing that a specific immune response is evoked by HIV during primary infection. This consists in the appearance of p24 Gag-specific T-helper responses and cytotoxic T lymphocytes (CTL) specific for viral antigens. The strength of the CTL response seems to be a key factor in determining the viral set-point, which in turn will predict the future outcome in the individual patient. This reactivity usually declines within the first months or years of infection, unless a potent antiretroviral treatment is initiated during the acute phase. Indeed, in this case, strong and persistent gag-specific T helper CD4 and CTL activity is maintained in a high proportion of subjects, whereas among patients having initiated highly active antiretroviral therapy (HAART) during the chronic phase the same proportion is around 5%, or even less in those with $CD4^+$ cell count below $250/mm^3$. Unfortunately, early treatment, though able to keep plasma viral load to undetectable levels and to maintain a vigorous anti-HIV immune response, seems unable to prevent the establishment of a latent reservoir of HIV, represented by resting $CD4^+$ T cells.

New strategies are being designed for patients with chronic infection. These include new immunotherapeutic approaches, such as HIV vaccination or cytokine therapy (Chun et al., 1999; Davey et al., 2000), which might enhance virus-specific immunity, allowing the control of any viruses released from the latent reservoir, and the use of structured treatment interruptions, which, besides possibly boosting the immune system may decrease the overall exposure to antiretroviral drugs.

The hope is that stimulation of HIV-specific responses may increase the number of patients that achieve undetectable RNA levels, delay the emergence of resistance, forgive less than perfect adherence, prolong duration and, possibly, allow discontinuation of treatment (because we may turn patients into "long-term nonprogressors").

CONCLUSION

Although the global solution to the further spread of HIV may still rest on the discovery of an effective prophylactic vaccine, the availability—at least in the western world—of improved antiretroviral drugs together with the development of innovative treatment strategies, raises the hope of changing HIV disease into a chronic condition with no or little impact on the life span of the many already infected. Challenges to more successful HIV therapy include improving adherence to drugs by enhancing their pharmacological and toxicity profiles, containing the development of drug-resistance, and developing effective immune interventions. In fact, with HIV eradication not at the immediate horizon, the evidence of a specific and effective cellular immune response against HIV point to the potential advantages of an immune manipulation.

The fact that the impressive therapeutic gains in the management of HIV disease benefit only 10% of the HIV-infected population in the world highlights the need to develop strategies to increase access to care and treatment for the remaining 90%. On a global scale, the main challenge will be to make broadly available prevention methods, life-saving treatments, and the results of scientific breakthroughs in care, on an equitable and affordable basis, to all HIV-infected persons throughout the continents. Actions in this direction should clearly foster a sharp decrease of price of antiretroviral drugs in developing countries, but also be based on the overall strenghtening of local health systems, on building the infrastructures, on education and training of healthcare providers, and, finally, on the reorientation of basic and clinical research to address issues of antiretroviral treatment in countries with limited financial resources.

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