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HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 INFECTION OF HUMAN MYELOID CELLS

By

Cynthia Ann Pise-Masison

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

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June 1994

To my family, especially my mom, Nellie Pise, and my husband, Daniel Masison, without whose constant love, support, and understanding I would not have been able to fulfill my dreams.

HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 INFECTION OF HUMAN MYELOID CELLS

A Thesis

by

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ABSTRACT

Infection with human immunodeficiency virus type 1 (HIV-1) results in a wide range of immunologic and hematopoietic abnormalities. The overall goal of this dissertation was directed toward obtaining a better understanding of the interactions of HIV-1 and myeloid cells in relation to the pathogenesis of AIDS. The human myelomonocytic cell line, HL-60, was used as a model system to determine if HIV-1 infects myeloid progenitor cells and subsequently, if infection affects their differentiation. HL-60 cells and the human prototypic T cell line, H9 were infected with three different HIV-1 isolates (IIIB, PM213, and NL4-3) which are known to infect T cells. All three isolates productively infected both H9 and HL-60 cells; however, HIV-1 antigen expression and cytopathicity was delayed by approximately 15 days in infected HL-60 cells compared H9 cells. To examine the effect of HIV-1 infection on myeloid differentiation, chronically infected HL-60 cells and clonal lines derived from them were induced to differentiate into either granulocytes by treatment with dimethyl formamide (DMF) or into monocytes by treatment with phorbol 12-myristate 13 acetate (PMA). By both cellular morphology and function, approximately the same percentage of treated, HIV-infected HL-60 cells differentiated into either granulocytes or monocytes as treated, control HL-60 cells. Taken together, these results indicate that HIV-1 infection does not affect the morphological or functional differentiation of HL-60 cells.

In an effort to understand the differences in the regulation of HIV-1 infection in myeloid versus T cells, the life cycle of NL4-3 was examined in HL-60 cells and H9 cells. Initially, NL4-3 replication was restricted in HL-60 cells compared to H9 cells. This restriction was overcome 15 days after infection by the generation of a viral isolate, NL4-3(M). NL4-3(M), harvested during the lytic phase of NL4-3 infection of HL-60 cells, caused cell death approximately 8 days after infection in both H9 and HL-60 cells. Although measurements of viral entry kinetics demonstrated that the timing of entry of NL4-3 and NL4-3(M) in HL-60 cells and NL4-3 in H9 cells was similar, a quantitative

polymerase chain reaction (PCR) analysis of newly reverse transcribed NL4-3 DNA in H9 and HL-60 cells revealed that NL4-3 infected H9 cells and NL4-3(M) infected HL-60 cells contain consistently higher amounts of newly reverse transcribed DNA than NL4-3 infected HL-60 cells. The delay in NL4-3 replication in HL-60 cells was further amplified by inefficient spread of the virus throughout the HL-60 culture as measured by RNA production and DNA integration suggesting that another step in the viral life cycle after reverse transcription was also restricted. These results suggest that the efficiency of NL4-3 replication in HL-60 cells is restricted at several steps in the viral life cycle. Further, these restrictions are overcome by the generation of a viral variant, NL4-3(M), which efficiently replicates in myeloid cells.

The tropism of NL4-3(M) was further characterized by testing its growth in monocyte-derived macrophages (MDM). Unlike NL4-3, NL4-3(M) productively infected MDM cultures. The ability of NL4-3(M) to infect macrophages was conferred by the envelope gene. This was demonstrated by the ability of the recombinant virus, NL4-3envA, which contains the envelope of NL4-3(M) in the context of the NL4-3 genome, to infect and replicate in MDM cultures. The envelope gene of NL4-3(M), however, did not confer ability to rapidly kill HL-60 cells. Together, these findings demonstrate that viral determinants controlling entry into MDM are different from the determinants controlling the cytopathic phenotype in HL-60 cells.

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CHAPTER I

INTRODUCTION

Introduction.

Human immunodeficiency virus type 1 (HIV-1) infection initiates a slowly progressive degenerative disease of the immune system, termed acquired immunodeficiency syndrome (AIDS). Based on virion morphology, genome organization, and pathogenic features, HIV-1 has been classified as a member of the lentivirus subfamily of retroviruses. Like other lentiviruses, HIV-1 has been shown to be complex compared to the oncoretroviruses (1). Since its discovery a remarkable amount of information about the molecular biology of HIV-1 has been determined which has provided insight into basic mechanisms underlying viral latency, gene regulation, and immune evasion. One aspect of AIDS is the generation of viral variants which is reflected in the characteristics of disease progression. The subject of this dissertation concerns the generation of viral diversity and its relationship to viral pathogenesis. To provide background information, these subjects are reviewed in detail below.

Entry of HIV-1 into Cells and Synthesis of Viral DNA.

HIV-1 infection of a cell is initiated when a virus particle binds a CD4 molecule (2). A second receptor or cofactor may be necessary to aid in viral entry (3-5). Entry occurs following direct fusion of virus and cell membranes (6-10), and is mediated by the envelope glycoprotein of the virus (11, 12). The envelope protein is composed of two subunits, glycoprotein 120 (gp120) and glycoprotein 41 (gp41). The exterior portion of the envelope protein, gp120, specifies binding to CD4 and is thought to play an important role in cellular tropism (13-21). Together gp120 and gp41 mediate the fusion of the viral and host cell membranes to allow viral entry (6).

Following internalization and partial uncoating of the virion to an enzymatically active nucleoprotein complex (22), the viral genome is converted from single stranded RNA to double stranded DNA by the viral-encoded enzyme reverse transcriptase (RT). RT is packaged with two full length viral RNAs and a cellular $tRNA^{lys}$ in the virus

particle and is thus poised for initiation of viral DNA synthesis. In fact, recent evidence indicates that partial reverse transcription can occur in the virus particle prior to cell entry (23). The primer for the minus strand DNA synthesis, $tRNA^{lys}$, binds specifically to a site near the 5' end of the RNA genome called the primer binding site (PBS). DNA synthesis initiates from the PBS and proceeds toward the 5' end of the viral RNA, the U5 and R regions. The ribonuclease H activity of RT removes sequences near the 5' end of the RNA template of the RNA-DNA hybrid which allows the single-strand RNA-DNA to "jump" and hybridize to R sequences at the 3' end of the viral genome. Once reprimed, the synthesis of minus strand DNA is then completed. Synthesis of the plus strand DNA is initiated from the RNA primer following nucleolytic digestion of genomic RNA by the RT RNase H (24). Using the minus strand DNA as a template, the plus strand is completed to form a linear DNA duplex. This reaction is typically completed within 6-8 hours after infection (25-29), at which time viral DNA can be found in the cytoplasm within a nucleocapsid protein structure termed the preintegration complex.

Integration of Viral DNA.

After active transport of the preintegration complex to the cell nucleus (30), the viral integrase protein, also packaged within the virus particle, mediates the insertion of the viral DNA into the host genome (25, 26). Although three forms of viral DNA (linear, one long terminal repeat (LTR) circles, and two LTR circles) can be found in the nucleus, evidence strongly suggests that only the linear form integrates into the genome of the host cell (31, 32). In most retroviral systems the circular DNA forms are short-lived, however, in HIV-1 infection, a substantial amount of HIV DNA exists in an unintegrated form, potentially contributing to the pathogenesis observed in HIV infection (33). *In vitro* studies of HIV-1 and MuLV integrase have revealed that it has three major activities: (1) endonuclease cleavage of the ends of the viral linear DNA (usually two nucleotides) in preparation for provirus formation, (2) cleavage of the host DNA, and (3) covalently

joining the cut ends of the viral and host DNA. Integration is thought to be an essential step in establishing a productive infection (34-39).

Transcription and Translation of HIV-specific mRNAs.

Once the HIV-1 proviral DNA is integrated, viral replication may enter either a latent, restricted phase, or a productive phase depending on the state of activation of the cell (40, 41). A number of *in vitro* experiments have shown that latent or chronic infections, where a low level of virus is produced, can be activated to produce increased viral expression by using mitogens (42, 43), heterologous viral infections (44-46), and cytokines (47-50). In an activated cell, host cell factors such as NFκB, Sp1, EBP 1, and UBP1 (51-55) which vary according to cell type, initiate viral transcription. This basal level of transcription allows production of genomic and messenger RNA (mRNA). The full-length viral RNA transcript contains multiple splicing sites which are acted on by the cellular mRNA splicing machinery. The multiply spliced viral mRNAs are exported from the nucleus to the cytoplasm where they are translated.

A key translation product of these multiply spliced RNAs is a viral encoded trans-activator protein, Tat, an 86 amino acid protein encoded by two exons that is essential for virus replication (56-59). HIV-1 gene expression is then regulated by an autostimulatory pathway involving interaction of Tat with the sequence contained within the LTR called TAR for trans-activating responsive region (60, 61). The Tat protein, made early in infection, accelerates the rate of viral transcription throughout the course of infection. Kao and coworkers (62) have also shown an accumulation of prematurely terminated transcripts in the absence of Tat, implicating it as an anti-termination factor. In addition to increasing the level of viral mRNA, Tat appears to increase the level of viral proteins synthesized in a manner disproportionate to the level of RNA increase, suggesting that Tat also affects the efficiency of message utilization (63). This could be accomplished by

stabilization, transport, or facilitating translation of the message. Thus the mechanism of transactivation by Tat may be due to an increase in the translation of viral mRNA (64, 65), and/or to an increase in the steady-state level of viral mRNA (66-69). Cellular proteins which bind to both Tat and TAR have been found; however, the role they play in Tat function and viral replication is still unclear (70-73).

Another multiply spliced RNA transcript encodes the Rev protein (74) which plays an important role in determining the fate of primary RNA transcripts. Rev protein is found in the nucleus and preferentially in the nucleolus of the infected cell (75, 76). In the absence of Rev, only multiply spliced viral RNA transcripts can be found in the cytoplasm while in the presence of Rev, singly spliced and unspliced viral mRNAs can be found in the cytoplasm (77-80). Rev binds to a complex stem-loop structure termed the Rev responsive element (RRE) found within the envelope coding region of the viral RNA (81-84). The binding of Rev to the RRE is thought to overcome an inhibitory effect due to cis-acting repressive sequences (CRS) present within the same mRNAs (85, 86). The exact mechanism and specific host cell factors involved are still unclear. However, the net effect of Rev activity is to permit accumulation of singly spliced and unspliced messages in the cytoplasm where they are translated to make the capsid and envelope proteins of the virus.

Assembly of the Virus Capsid.

The capsid protein is synthesized as a polyprotein precursor from the *gag* gene (87). The replicative enzyme precursor, polymerase (*pol*), is expressed initially as a Gag-pol fusion protein. In the gag-pol mRNA the two genes overlap by 241 nucleotides with the translational reading frame of the *pol* gene shifted by -1 nucleotide with respect to *gag*. A ribosomal frameshift event, mediated by a short sequence located in the *gag-pol* overlap, allows synthesis of the Pol polyprotein in a ratio of 20:1 (88, 89, 310). The Gag and Gag-pol polyproteins assemble at the inner surface of the cell membrane. A myristic acid

attached to the amino terminus of the capsid precursor allows its insertion into the cell membrane (90, 91). Two strands of genomic viral RNA bind to capsid precursor protein via interaction of the packaging sequence, Psi, in the 5' end of the RNA with the cysteine-histidine box of the capsid proteins (92-95). The complex of genomic RNA, Gag and Gag-pol polyproteins assemble into a closed spherical particle which buds from the cell membrane containing the viral envelope glycoproteins. Late maturation events include proteolytic cleavage of the capsid protein and the pol precursor (96) by the viral protease protein. The virus particle is finally released from the cell surface by an unknown membrane cleavage mechanism completing a round of viral replication.

Viral Regulatory Proteins.

There are at least two viral regulatory proteins that are thought to act late in the virus life cycle to facilitate virus release and to increase viral infectivity. One of these proteins is the Vif protein made from a singly spliced mRNA that accumulates late in infection (97, 98). Studies involving viruses containing a mutant *vif* gene, demonstrate that Vif^- viruses are defective for cell-free transmission (99-103), but no structural abnormalities of the virion have been reported. In addition, the growth ability of the Vif mutants has been reported to vary depending on the $CD4^+$ target cells (36, 101, 104-110). Although the mechanism by which Vif acts is unclear, recent reports suggest that Vif interacts with the envelope protein in virions, directly or indirectly, to enhance viral infectivity in a cell-dependent manner (107, 111). Vif has also been reported to have an effect on viral DNA synthesis where inefficient viral DNA synthesis correlated with restricted replication of Vif^- viruses (108, 112).

A second protein that assists in viral maturation is the integral membrane phosphoprotein Vpu (113). Although Vpu is synthesized in amounts similar to those of the envelope glycoprotein in infected cells, it has not been detected in virions (114, 115). Comparison of the replication of infectious molecular clones of Vpu^- and Vpu^+ mutants

demonstrated that Vpu is involved late in viral replication. A decrease in extracellular virus with increases in levels of cell-associated virus proteins was demonstrated in cells infected by Vpu$^-$ viruses (116-118). Furthermore, increased intracellular budding and aberrant budding structures at the plasma membrane were observed (116). Recent evidence indicates that Vpu also induces rapid degradation of CD4 (119, 120) and enhances processing of gp160 (119, 121) suggesting that Vpu may increase viral production by disrupting CD4-gp160 complexes formed in the endoplasmic reticulum of the cell. Recently, Geraghty and coworkers (122) have also found evidence that Vpu has a function involving particle release that is independent of CD4 or gp160 expression.

Another small regulatory protein, the only one so far demonstrated to be packaged within the virus particle (approximately 100 copies/virion), is the Vpr protein (123-127). Vpr is made from a singly spliced mRNA that accumulates late in infection (128-131). By a yet undefined mechanism, Vpr is thought to increase the rate of replication of HIV, perhaps by increasing gene expression from the HIV-1 promoter (129-131). Since the protein is present in virions and is subsequently found in the nucleus, Vpr is suggested to have a role early in infection before new viral protein synthesis occurs.

The last known regulatory protein encoded by the HIV-1 genome is Nef (87, 132). Nef mRNA is detected early in infection along with mRNA for Tat and Rev (133). However, unlike Tat and Rev, Nef is a cytoplasmic protein which appears to be partly associated with the membrane (134, 135). The *nef* gene gives rise to two translation products of 25 and 27 kDa. The 27 kDa protein has a myristylation motif which probably allows interaction with cell membranes. The 25 kDa protein, which is not always detectable in HIV-infect cells (135), is produced from internal initiation and thus is not myristylated. Because of earlier evidence of its inhibitory effect on the HIV-1 LTR and virus replication in cell culture, Nef was considered to be a negative regulatory protein involved in establishing viral latency (136, 137). Recent reports demonstrate, however, that Nef may be involved in efficient viral replication (138, 139) and in the development of

AIDS in monkeys (140). Kestler and coworkers (140) found that a provirus with a stop codon in Nef replicated *in vivo* only after selection for the removal of the stop signal. This indicates that although Nef appears unnecessary for *in vitro* propagation of HIV-1, it is likely to have a role in viral pathogenesis *in vivo*. While the mechanism by which Nef acts is unknown, recent reports suggest that Nef may be involved in signal transduction and viral and/or cellular gene regulation. Although the evidence that Nef binds purine nucleotides and is capable of autophosphorylation *in vitro* (141-143) has been met with some controversy (144, 145), these data suggest a possible regulation of Nef activity by phosphorylation. In addition, being a myristylated protein, Nef may interact with cell membranes and/or membrane proteins in a signal transduction cascade. Furthermore, gel retardation analysis shows that Nef interacts with nuclear factors associated with promoter elements (146), is capable of inducing down regulation of CD4 from the cell surface (146, 147), and is capable of blocking induction of interleukin 2 mRNA (148). These data suggest that Nef may also act at a transcriptional level.

Our understanding of the contribution of each of the virus' structural and regulatory genes to the complex life cycle of the virus in natural infections is incomplete. It is clear that HIV-1 is an extremely complex virus with genes that may facilitate latent viral infection, restricted chronic viral replication, or high levels of virus production and cell death. The interaction of HIV-1 regulatory proteins with each other and with cellular factors can allow a switch from one pathway to another. The interactions of cellular factors with the viral genome are thought to play a role in the maintenance of the variable asymptomatic phase in infected individuals and the subsequent progressive immunological deterioration.

Viral Diversity and Its Relationship to Disease.

One characteristic feature of lentiviruses, in particular HIV-1, is genetic variability. Variation of HIV-1 isolates from one patient to another has been shown by restriction endonuclease analyses of molecularly cloned proviruses (33, 149, 150). Comparison of nucleotide sequences of different HIV-1 isolates (21, 151-158) demonstrated that variation exists throughout the viral genome, with the region of greatest variability occurring in the envelope gene (151, 159). The envelope gene is composed of both conserved and hypervariable regions. The third variable region (V3) of the envelope gene, and in particular the V3 loop, is believed to be a major determinant of viral pathogenesis. The V3 region has been demonstrated to be involved in macrophage versus T lymphocyte tropism, viral entry, and syncytia formation (13-16, 18-20). Recent evidence has also implicated other regions of the envelope protein, as well as other viral genes, including *vif*, *nef*, *vpu*, and *gag* (103, 138, 160-168), in influencing tropism and cytopathicity.

HIV-1 variants show distinct biological features including replication kinetics, serum neutralization, cell tropism, and cytopathic effect (21, 112, 169-175). Viruses isolated from asymptomatic patients replicate slowly in peripheral blood mononuclear cells (PBMC's) and very inefficiently, if at all, in transformed cell lines, while viruses isolated from patients with severe immunodeficiency replicate rapidly and efficiently in PBMC's and in cell lines (152). In the early stages of the disease, primarily non-syncytia inducing (NSI), macrophage-tropic viruses can be isolated from infected individuals. As the disease progresses, syncytia-inducing (SI), T lymphocyte-tropic viruses are primarily isolated (152, 178, 180). The significance of macrophage tropic HIV-1 variants during the early stages of disease is further demonstrated by primary isolation studies on a donor-recipient pair in which virus was accidentally transmitted (176). In the donor, a terminal stage HIV-infected patient, a low frequency of macrophage tropic HIV isolates could be detected, while in the recipient, a high frequency of macrophage tropic isolates could be

demonstrated. These viruses may be adapted for survival in early HIV-1 infection when the immune response is thought to be most effective.

Recent evidence strongly suggests that the changes in the biological features of the virus with time (175, 177, 178), as well as increased virus load (179) are signs of increased virulence in the host. In addition, in about half of the individuals progressing to AIDS, a conversion from clones of the NSI to the SI phenotype can be observed (180). These findings together with the finding that macrophage tropism is regained by some late-stage SI clones (178) further supports the notion of increased virulence during the course of HIV-1 infection. Taken together, these results underscore the importance of defining the mechanisms involved in HIV diversity.

Mechanisms of Viral Diversity.

Viral genomic changes are known to arise by several mechanisms. One mechanism involves changes due to the viral RNA polymerase which has a high error rate and lacks proofreading function (181). While mutation rates for all retroviruses as a group are high (182, 183), the error rate of HIV-1 RT has been demonstrated to be approximately ten times higher than that of other retroviruses (182, 184, 185). Recent studies (186) suggest that HIV-1 RT is able to extend primers that contain a mismatched 3' terminus more efficiently than mammalian DNA polymerase. This function is required for processive synthesis after misincorporation. In addition, changes can arise during RNA synthesis by RNA polymerase II (Pol II) after integration. The fidelity of RNA synthesis by Pol II is unknown, but since there is no known editing function, it is likely to be similar to that of viral RNA polymerases (309). Another mechanism of generating viral genomic changes involves major substitutions in viruses that are introduced by viral recombination events occurring between two viral genomes or between viral and cellular sequences (187, 188). Recombination between retroviral genomes requires one round of viral replication in which one of each parental RNA genome is packaged into the same virion resulting in a

heterozygous particle (1, 189, 190). After infection of a new cell, both RNAs are copied, at least in part, into DNA by RT, at which point, recombination can occur. A third mechanism for generating retroviral diversity is rearrangement. Duplications and large and small deletions are believed to arise by template slippage, mispriming at direct repeats during reverse transcription, homologous recombination between direct repeats, or a pathway independent of homologous recombination (191).

The Effect of HIV-1 Infection on Hematopoiesis.

The most prominent feature of AIDS is the progressive depletion of $CD4^+$ T lymphocytes, resulting in profound immunosuppression. This HIV-induced immunosuppression renders the body highly susceptible to opportunistic infections and neoplasms (40, 192-199). HIV-1 is not only capable of infecting and killing T lymphocytes (192, 200), but is also capable of replicating in other cell types such as cells of the monocyte/macrophage lineages (201-208). In addition, abnormalities of the peripheral blood and bone marrow also have been described in AIDS patients. In order to investigate the direct involvement of HIV-1 infection in the pathogenesis of these abnormalities, several investigators have studied infection of hematopoietic progenitor cells. Impairment of the growth of hematopoietic cells which are precursors to the myeloid and lymphoid lineage could have some effect on the pathogenesis of the hematological alterations, as well as in the progressive reduction of $CD4^+$ T lymphocytes seen in AIDS patients.

The altered regulation of hematopoietic cells may be due to a combination of HIV-1 induced mechanisms. Bone marrow cells from HIV-infected individuals, free of autologous sera, exhibit reduced colony formation *in vitro* (209, 210). In addition, proliferation of granulocyte-macrophage progenitor cells from HIV-infected patients is reduced significantly as compared to controls (211). Direct HIV-1 infection of monocytic and megakaryocytic precursors and promonocytic cell lines *in vitro* has also been shown

(212-218). Furthermore, it has been demonstrated that the growth of the hematopoietic progenitors ($CD34^+$ cells) is inhibited by HIV-1 infection in the absence of complete viral replication (219), perhaps due to soluble viral factors such as gp120 (308). Consistent with this, viral DNA could not be detected by polymerase chain reaction (PCR) analysis in purified $CD34^+$ cells from 13 HIV-infected individuals whose progenitor cells showed impaired growth *in vitro* (220).

In addition to having a direct effect on the growth of hematopoietic progenitor cells, HIV-1 infection may also affect their growth by antibody-mediated killing of progenitor cells (221) or production of inhibitory factors by infected bone marrow mononuclear cells (211). Conflicting reports as to the role of HIV-1 in altered cytokine production by infected bone marrow stromal cells or T lymphocytes makes the contribution of cytokines to pathogenesis unclear. While some investigators have reported that HIV-1 infection stimulates cytokine production which, in turn, can stimulate HIV-1 gene expression (47-50), others failed to find high levels of cytokine expression induced by either viral infection or addition of viral components to normal peripheral blood mononuclear cells and purified monocytes (222-224). It is clear that HIV-1 directly affects the growth of hematopoietic progenitor cells, although further investigation is needed to elucidate the mechanisms involved in their derangement during infection. Moreover, HIV-1 could alter the hematopoietic compartment indirectly, through autoimmune mechanisms, enhanced production of soluble factors, or decreased production of growth factors by infected stromal cells.

Since T4-lymphocytes play an important role in the immune system and interact with monocytes, macrophages, cytotoxic T cells, natural killer cells, and B cells, it is clear that depletion of T4-lymphocytes may result in several immunological deficits which could ultimately lead to the opportunistic infections characteristic of AIDS. While the T4-lymphocyte is a reservoir for the virus in the bloodstream, the monocyte-macrophage plays a major role in HIV-1 persistence during early infection and dissemination of the

virus to compartments outside the peripheral blood. Researchers have shown that HIV-1 can infect peripheral blood monocytes (202, 204, 206), alveolar macrophages (225) and established monocytic cell lines (213, 214). HIV-1 has also been cultured from or detected in monocyte-macrophages from the blood (204), lung (202), and brain (199, 202, 226) of HIV-infected patients. Furthermore, HIV-1 infection of monocyte-derived-macrophages appears to be persistent and does not result in significant cell death or syncytia that occurs after infection of T cells (193, 203, 208, 225). The apparent resistance of these cells to HIV-1 induced cytopathicity makes them important intracellular reservoirs for virus.

HL-60 Cells as a Model System for HIV-1 Infection of Myeloid Cells.

The research presented in this dissertation was directed toward obtaining a better understanding of the mechanisms involved in HIV-1 pathogenesis by examining the effect of HIV-1 infection in human myeloid cells and focused primarily on: (1) the effect of HIV-1 infection on progenitor cell differentiation, (2) a comparison of the replication of HIV-1 in T cells versus myeloid cells, and (3) the role of viral variants in tropism and cytopathicity. The human promyelocytic leukemia cell line, HL-60 was used as a model system to address these questions. HL-60 cells are a bipotential myeloid cell line which can be chemically induced to differentiate into granulocytes or macrophages. This system provided the opportunity to examine the effects of HIV-1 infection on myeloid precursor cell differentiation, the results of which are described in Chapter II.

Despite the fact that HIV-1 infection of HL-60 cells does not affect granulocytic or monocytic differentiation, infection does cause cytopathicity in HL-60 cells. Cell death, however, is delayed significantly in HL-60 cells as compared to the human T cell line, H9 (Chapter II). Although HIV-1 replication has been reported to differ in monocytes and T cells (193, 208, 225, 227), the mechanisms which drive these differences have not yet been defined. In order to determine what factors affect HIV-1 replication in myeloid cells, the

life cycle of the lymphotropic HIV-1 isolate, NL4-3, was compared in HL-60 and H9 cells. The results of these studies are presented in Chapter III.

Lastly, I undertook an analysis of the generation of a viral variant, NL4-3(M), which was isolated during the lytic infection of NL4-3 in HL-60 cells and the role of NL4-3(M) in viral pathogenesis. The life cycle of NL4-3(M) was characterized during infection of both H9 and HL-60 cells and the results from this study are described in Chapter III. In addition to acquiring an increased cytopathic phenotype on the monocyte precursor cells, HL-60, NL4-3(M) acquired macrophage tropism. Experiments describing this extended tropism are presented in Chapter IV. Taken together, the results in this dissertation demonstrate that during HIV-1 infection of myeloid precursor cells, a viral variant is produced which is capable of efficient replication in these cells as well as in differentiated macrophages. The change in biological phenotype of NL4-3(M) which overcomes the restrictions to replication of NL4-3 in HL-60 cells is specifically selected by growth in HL-60 cells and is inherited stably.

CHAPTER II

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTED HL-60 CELLS ARE CAPABLE OF BOTH MONOCYTIC AND GRANULOCYTIC DIFFERENTIATION

(Pise, C. A., Newburger, P. E., and Holland, C. A. 1992. Human Immunodeficiency Virus Type 1 Infected HL-60 Cells are Capable of Both Monocytic and Granulocytic Differentiation. J. Gen. Virol. 73:3257-3261)

INTRODUCTION

Several lines of evidence suggest that monocytes infected by HIV-1 are important to the pathogenesis of AIDS (227, 228). HIV-1 has been shown to infect established monocytic and promonocytic cell lines and primary peripheral blood monocytes *in vitro* (194, 202, 204, 208, 215, 224, 229-232). Viral RNA, proviruses, and viral antigens have been demonstrated in brain macrophages, follicular dendritic cells in lymph nodes, and in cells with macrophage markers of the skin and lungs of AIDS patients (199, 225, 233, 234). In addition, HIV-1 can be isolated from peripheral blood monocytes and bone marrow of AIDS patients (218, 235, 236). Thus it has been suggested that monocytes/macrophages serve as reservoirs of HIV-1. As mediators of viral dissemination, they may be important to the progression of the disease.

HIV-1 infection affects monocyte/macrophage function *in vivo*. Functional alterations of monocytes/macrophages from AIDS patients include significantly reduced phagocytic and chemotactic activities (237), monocyte-dependent T-cell proliferation (238), Fc receptor function (239), and accessory cell function (240, 241).

HIV-1 can also infect myeloid progenitor cells (212), and growth of hematopoietic progenitor cells of AIDS patients can be suppressed by anti-HIV antibodies (221). Bone marrow cells from HIV-infected individuals, free of autologous sera, have been shown to have reduced colony formation *in vitro* (209, 210). In addition, proliferation of granulocyte-macrophage progenitor cells from HIV-1 infected patients is significantly reduced as compared to controls (211). The data from studies on bone marrow suppression and the dysfunction of infected monocytes suggest that both activation and differentiation of monocytes may be altered by HIV-1 infection.

In this study, I have used HL-60 cells to examine the effect of three different HIV-1 isolates on granulocytic and monocytic differentiation of a myelomonocytic precursor. I have chosen three isolates of HIV-1 (NL4-3, IIIB, and PM213) that grow to high titer in both H9 and HL-60 cells to compare the cytopathic effect of the virus isolates on both cell types and to determine the effect of HIV-1 infection on granulocytic and monocytic differentiation of HL-60 cells.

MATERIALS AND METHODS

Cell culture conditions. The human $CD4^+$ lymphoblastoid cell line, H9 (provided by Dr. R. Gallo, through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, Bethesda, MD), the human myelomonocytic cell line, HL-60 (242, 243) (a gift from Dr. Robert Gallo), and the human promyelocytic cell line, PLB985 (251) were maintained at densities between 2×10^5 and 2×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, supplemented with penicillin (100 units/ml)-streptomycin (100 micrograms/ml). Cell counts were performed using a hemacytometer and viability was determined by trypan blue exclusion.

Viruses and infection. Cell-free virus stocks of HIV NL4-3,(a gift from Dr. Carel Mulder, 104), IIIB, and PM213 (231) were prepared in the T lymphoblastoid cell line, H9. The infectious titers of the virus stocks were determined by infecting C8166 cells, a HTLV-I immortalized cell line, (244). Twenty-four hours after infection the number of cells that react with a pooled patient serum and fluoresceinated IgG antibody (Sigma Chemical Co, St. Louis, MO) were analyzed.

Cells were split on the day before infection. Cultures of H9 or HL-60 cells were infected at a multiplicity of infection of 0.2 tissue culture infectious doses (TCID)/cell in the presence of 2μg/ml polybrene. Twenty-four hours after infection, the cells were pelleted, washed with PBS, and maintained in RPMI 1640 containing 10% fetal bovine serum seeded at 1×10^6 cells/ml daily throughout the remainder of the experiment. For virus stocks, cell-free supernatant was harvested daily and stored at -70^oC. Chronically infected cultures (>30 days after HIV-1 infection) were used for differentiation analysis. Productive infection was confirmed by reverse transcriptase (RT) activity.

Indirect immunofluorescence. The percentage of infected cells expressing HIV-1 surface antigens was determined by indirect immunofluorescence (IFA) using an HIV-1

positive pooled patient serum as the primary antibody. Briefly, 1 x 10^5 cells were applied to a glass slide, air-dried, and fixed in methanol for 5 minutes. The fixed cells were incubated with HIV-1 positive pooled patient serum at 37°C in 5% CO_2 for 40 minutes, washed with PBS, incubated with fluorescein-conjugated goat anti-human IgG at 37°C in 5% CO_2 for 40 minutes, washed with PBS, and stored at 4°C in PBS:glycerol (1:1). The number of fluorescent cells per 1000 cells was determined using a fluorescence microscope.

Cell surface CD4 expression was analyzed by fluorescence-activated cell sorting (FACS). Cells (2 x 10^6) were incubated with the primary antibody OKT4, an anti-CD4 antibody (OrthoDiagnostics Systems Inc., Raritan, NJ), and then stained with fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Co, St. Louis, MO).

Single cell cloning. Uninfected HL-60 cells and H9 and HL-60 cells chronically infected with HIV IIIB were cloned by plating the cells in a 96 well plate at a density of 0.5 cells/well. Cultures were then expanded and stained for HIV-1 expression by IFA. A second round of cloning was done on HIV-1 RT positive cultures.

Differentiation. HL-60 cells were induced to differentiate into granulocytes or monocytes. Granulocytic differentiation was induced in HL-60 cultures (cell density = 1 x 10^6 cells/ml) by addition of 80mM dimethyl formamide, DMF (Sigma Chemical Co., St. Louis, MO). Cell differential counts were performed on Wright-Giemsa stained, induced cells. Four stages of granulocytic differentiation were distinguished: promyelocytes, myelocytes, metamyelocytes, and polymorphonuclear cells (PMNs) (245) by their morphology.

Superoxide production of granulocytes (246) was monitored spectrophotometrically following the reduction of nitroblue tetrazolium (NBT) (247). The ability of DMF-treated HL-60 cells to generate superoxide (O_2^-) was compared to that of normal human PMNs. On day 6 after treatment, 1 x 10^6 cells from each culture were washed with PBS and suspended in 1.0ml reaction buffer containing PBS, glucose, 0.08% NBT in normal saline,

1mM KCN, and 1mg/ml phorbol 12-myristate 13 acetate (PMA). Background values were obtained by suspending 1 x 10^6 cells in reaction buffer without PMA. Samples were incubated at 37°C with shaking for 15 minutes. The reaction was stopped by addition of 10.0ml of 0.5N HCl. Cell were pelleted by centrifugation (800g for 15 minutes) and lysed with 1.0ml of dioxane. Cellular debris was pelleted (300g for 15 minutes) and the A_{515nm} of each supernatant was determined.

Monocytic differentiation was induced in cultures by addition of 62ng/ml PMA with cells at 1 x 10^6 cells/ml. Cultures were monitored from 1 to 3 days after treatment. Monocytic differentiation was indicated by cellular adherence and non-specific esterase staining (248).

Reverse transcriptase activity. RT activity was measured as described by Kunsch and Wigdahl (249). Briefly, 1.0ml of cell-free supernatant from mock or HIV-1 infected cultures were centrifuged at 12,000g for 1 hour, the virus pellet was suspended in 50μl of reaction mixture (50mM Tris-HCl, pH8.0; 5mM $MgCl_2$; 150mM KCl; 0.5mM EGTA; 5mM dithiothreitol; 0.3mM glutathione; 0.05% Triton-X; 50μg/ml poly(rA)oligo(dT) [Pharmacia, Piscataway, NJ]; and 50μCi/ml [^{3}H]thymidine triphosphate), and incubated at 37°C for 1 hour. Reactions were stopped by spotting them onto sodium pyrophosphate soaked filters. The filters were air dried, washed with cold 5% TCA 3 times followed by 95% ethanol, and counted by liquid scintillation spectrometry. All determinations were performed in duplicate.

CD4 DEPENDENT ENTRY. Cells (5 x 10^5) were incubated with 250ng/ml of Leu3a (Becton-Dickinson, Mountain View, Ca), an anti-CD4 antibody or medium for 10 minutes prior to the addition of virus at an MOI of 0.5 C8166 TCID/cell. The infection was done in the presence of 2μg/ml polybrene. Twenty-four hours after infection the cells were centrifuged (500g), washed with PBS, and suspended in RPMI 1640 containing 10% FCS at a cell density of 1 X 10^6 cells/ml. The cultures were monitored daily for viability by trypan blue exclusion and viral antigen expression by IFA.

RESULTS

HIV-1 is cytopathic to both H9 and HL-60 cells. The cytopathic effect of HIV-1 infection on HL-60 cells was compared to the cytopathic effect on H9 cells. Each cell line was infected at an MOI of 0.2 TCID/cell with each of the three HIV-1 isolates (IIIB, PM213, and NL4-3). The three viruses caused a rapid death of H9 cells (Fig II-1). Approximately 90% of the infected H9 cultures died within 12 days after infection. The mean survival time (MS_{50}), defined as the time at which the culture was 50% viable, was approximately 7 days for H9 cell cultures and was independent of the virus isolate tested. The percentage of infected H9 cells expressing HIV-1 surface antigens was determined by IFA. Fig II-2A shows the results of infecting H9 cells with NL4-3. Similar results were obtained with IIIB and PM213 (data not shown). As the percentage of viable cells decreased, the proportion of HIV-1 antigen expressing cells and the level of detectable RT increased (Fig II-2A).

The effect of HIV-1 infection on HL-60 cells was very different. After infection, the cells continued to proliferate. Greater than 90% of the culture remained viable for the first 10 days after infection (Fig. II-1). During this time 2 to 5% of the cells were positive for HIV-1 antigen expression (Fig. II-2B). Approximately 15 days after infection, the viability of the culture began to decrease (Fig. II-1). By 30 days the majority of the cells were dead. The MS_{50} for infected HL-60 cells was approximately 22 days. Both HIV-1 antigen expressing cells and RT activity present in the culture medium increased as the viability of the culture decreased (Fig. II-2B). Each of the three HIV-1 isolates killed HL-60 cells with similar kinetics (Fig. II-1) and results shown in Fig. II-2B are typical of infection with either NL4-3, IIIB, or PM213.

CD4 expression on HL-60 and H9 cells. Since it was not clear whether HIV-1 infection of HL-60 cells was mediated through CD4-HIV-1 envelope interactions, HL-60

cells were pre-incubated with Leu3a, an anti-CD4 antibody which blocks HIV infection of H9 cells (Becton-Dickinson, Mountain View, CA). Leu3a completely blocked HL-60 infection for 25 days post inoculation (Table II-1). Therefore infection of H9 and HL-60 cells most likely occurs via CD4-HIV-1 envelope interactions.

The difference in the observed kinetics of cell death of HIV-1 infected HL-60 and H9 cells could be due to the differences in the percentage of cells that express CD4 on the cell surface. This was analyzed by FACS. The percentage of CD4 positive cells was similar in H9 and HL-60 cultures (>85% of each culture was $CD4^+$; see Table II-1). In addition, the fluorescence intensity of HL-60 and H9 cells differed by 13% (based on median channel intensity), indicating that the number of receptors/cell differed by less than 10-fold. Others have shown that a 10-fold change in receptor number does not change the rate of spread of HIV-1 (250). These data support the conclusion that the differences in the observed kinetics of cell death of HIV-1 infected HL-60 and H9 cells are due neither to differences in the percentages of cells that express CD4 on their surface nor to the number of CD4 molecules/cell.

Differentiation of myeloid cells. HL-60 cells differentiate into granulocytes when treated with DMF or into monocytes when treated with PMA. I have used this characteristic to determine if HIV-1 infection affects the bipotential differentiation of HL-60 cells. HL-60 cell cultures were infected with HIV-1 IIIB, NL4-3 or PM213. On day 36 after infection, when the cultures have a measurable level of HIV-1 antigen expression, granulocytic differentiation was induced by addition of DMF. On day 6 after induction, cell differential counts were performed on Wright-Giemsa stained cells. The percentage of cells at each stage of granulocytic differentiation was scored on the basis of morphology as described in Materials and Methods. It was shown that the percentage of cells in each stage of differentiation in uninfected and infected HL-60 cell cultures was approximately the same (Table II-2). Thus granulocytic differentiation of chronically infected HL-60 cells is indistinguishable from uninfected HL-60 cells.

To study a more homogenous population of cells, a series of subclones of either HL-60 cells or chronically infected HL-60 cells (IIIB) were established. Infected clones were isolated by performing two sequential limiting dilutions (0.5 cells/well). Clones were isolated at a frequency of approximately 5% in the first dilution experiment (5 of 96) and 9% (18 of 192) in the second serial dilution experiment. All clones examined formed syncytia when co-cultivated with C8166 cells (15 lines). Of 10 clones that were tested for RT activity, nine were RT positive. The cloning efficiency and tests of viral expression suggest that the lines are clones and represent a population of infected cells. The percentage of IFA^+ cells (Table II-2) suggests, in addition, that only a proportion of the cells in the population express viral antigens. This is consistent with a previous report that HIV-1 DNA in HL-60 cells is extrachromosomal and therefore is not faithfully transmitted during clonal expansion (230).

One control uninfected HL-60 clone, HL-60 C1 (Table II-2) and two HL-60 IIIB clones, HL-60 IIIB-1 and HL-60 IIIB-2, were chosen for further analysis. The two HIV infected clones were determined to be HIV-1 positive by FACS analysis and RT activity. Like the parental uninfected HL-60 cell line, HL-60 IIIB-1 and HL-60 IIIB-2 cells were predominantly promyelocytic as determined by Wright-Giesma staining, and were used with three chronically infected HL-60 populations to examine differentiation of HIV-infected myeloid cells. When HL-60 IIIB-1 and HL-60 IIIB-2 were treated with DMF their differentiation was similar to uninfected or chronically infected HL-60 cells (Table II-2).

To determine whether these results were unique to HL-60 cells, identical experiments were performed with another human myelomonocytic cell line, PLB985 (251). These cells are slightly more primitive than HL-60 cells but are also bipotential and differentiate in response to DMF and PMA (251). The MS_{50} for HIV-1 infected PLB985 cells was approximately 20 days (Fig. II-1). Although there was a slight increase in the rate of cell death of infected PLB985 cells in comparison to infected HL-60 cells, both the

kinetics of HIV-1 infection (Fig II-1) and differentiation (Table II-2) were similar to those of infected HL-60 cells.

To test the function of differentiated HL-60 cell cultures infected with HIV-1, superoxide production was measured. Superoxide production was similar in uninfected and infected HL-60 cells (Table II-2). Therefore HIV-1 infection affects neither the granulocytic differentiation potential nor the functional capability of differentiated HL-60 cells.

HL-60 cultures persistently infected with HIV-1, clonal lines of infected HL-60 cells established from the persistently infected cultures, and HIV-1 IIIB infected PLB985 cells were capable of differentiating into monocytes by addition of PMA. Greater than 90% of both the infected and uninfected cultures became adherent after differentiation. An equally high proportion of cells (>89%) in each culture stained positive for the monocyte specific enzyme, non-specific esterase (248). The results indicate that HIV-1 infection affects neither differentiation of HL-60 cells into monocytes nor the production of a monocyte specific enzyme.

DISCUSSION

HL-60 cells can be induced to differentiate into macrophages by PMA and into granulocytes with DMF. I have shown that HIV-1 infection of HL-60 cells does not induce differentiation of these cells. Neither does HIV-1 infection abolish or inhibit the response of HL-60 cells to either PMA or DMF treatment. This is in contrast to recent evidence that HIV-1 infection of the promonocytic cell line, U937, (a more differentiated cell line than HL-60) induces differentiation of these cells and that the stage of cellular differentiation of U937 cells may determine the pattern of virus replication (214) and production (213). In addition, Roulston *et al.* (252) have reported that chronically infected lines of PLB-IIIB cells are more monocytic than the parental PLB985 cells and non-responsive to granulocytic differentiation induced by dibutyryl cAMP. The difference between these results and my results may be due to a difference in the cells examined or the agent used to induce differentiation. Both of the previous studies were performed using cell lines established from populations of cells that survive an HIV-1 infection. I induced differentiation in chronically infected HL-60 and PLB985 cells or chronically infected cloned HL-60 cell lines by treatment with DMF. Alternatively, the stage of myelocytic differentiation of hematopoietic cells may determine whether HIV-1 infection affects differentiation.

Granulocytopenias have been reported in up to two-thirds of patients with AIDS/ARC (236). One possible explanation for the reduction of granulocytes in HIV-infected individuals is that granulocytic precursor cells are unable to differentiate properly. This system is unique in that both granulocytic and monocytic differentiation of HIV-1 infected precursor cells could be examined. Chronically infected HL-60 cells, when induced to differentiate into granulocytes exhibit a pattern of maturation similar to that of uninfected cells. In addition, these cells exhibit normal superoxide production. These *in vitro*

findings suggest that HIV-1 infection of granulocytic precursor cells may not contribute to the granulocytopenia seen *in vivo*. Alternatively, the *in vivo* biology of monocyte/macrophages and granulocytes may be complex and agents other than those present in culture may effect the differentiation of infected cells.

Although a normal pattern of differentiation was observed for HIV-1 infected myeloid precursor cells (HL-60) *in vitro*, this does not rule out the possibility that *in vivo* the differentiation of these cells may be altered. This system examines lineage-committed progenitor cells and not multipotent cells or the self-renewing hematopoietic stem cell. HIV-1 infection of either of these cell types may result in abnormal hematopoiesis. A direct role for the virus in bone marrow suppression is suggested since HIV-1 can infect and replicate in myeloid ($CD34^{+}$) progenitor cells (212). Recent reports indicate that the number of hematopoietic progenitor cells is reduced in AIDS/ARC patients (209, 211, 239), infection of human $CD34^{+}$ cells causes a loss in the ability of the cells to give rise to colonies of differentiated progeny (253), and that the reduction in CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM is related to the stage of the disease (254). A similar decrease in hematopoietic progenitor colony formation is also observed during SIVmac (255) and FIV (256) infections. Bone marrow accessory cells (211), immune mediated suppression (221), and production of inhibitory molecules and cytokines from infected accessory cells may all play a role in the abnormal hematopoiesis observed in AIDS patients.

FIGURES AND TABLES

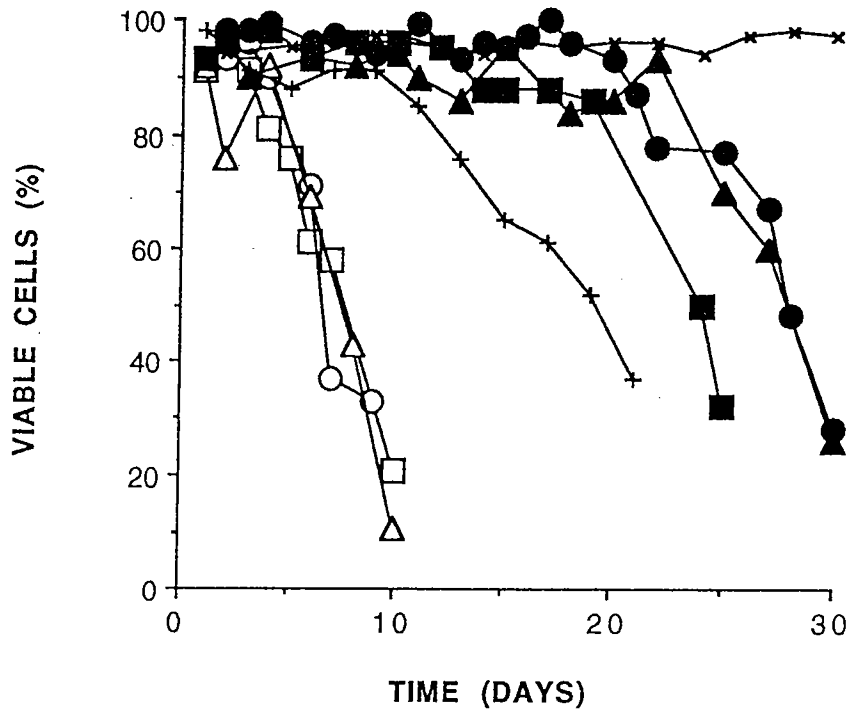

FIGURE II-1. **Infection of human myeloid cells with HIV-1.** Logarithmically dividing H9, HL-60, and PLB985 cells were infected with HIV IIIB, HIV NL4-3, or HIV PM213 at a multiplicity of infection of 0.2 TCID/cell in the presence of 2 μg/ml polybrene. The mean of the data from three independent experiments is plotted in comparison to uninfected HL-60 cells (X). Data from HL-60 and H9 cells are plotted using closed and open symbols, respectively (-□-, NL4-3; -O-, PM213; -Δ-, IIIB). Data for PLB985 cells infected with IIIB are plotted using the + symbol.

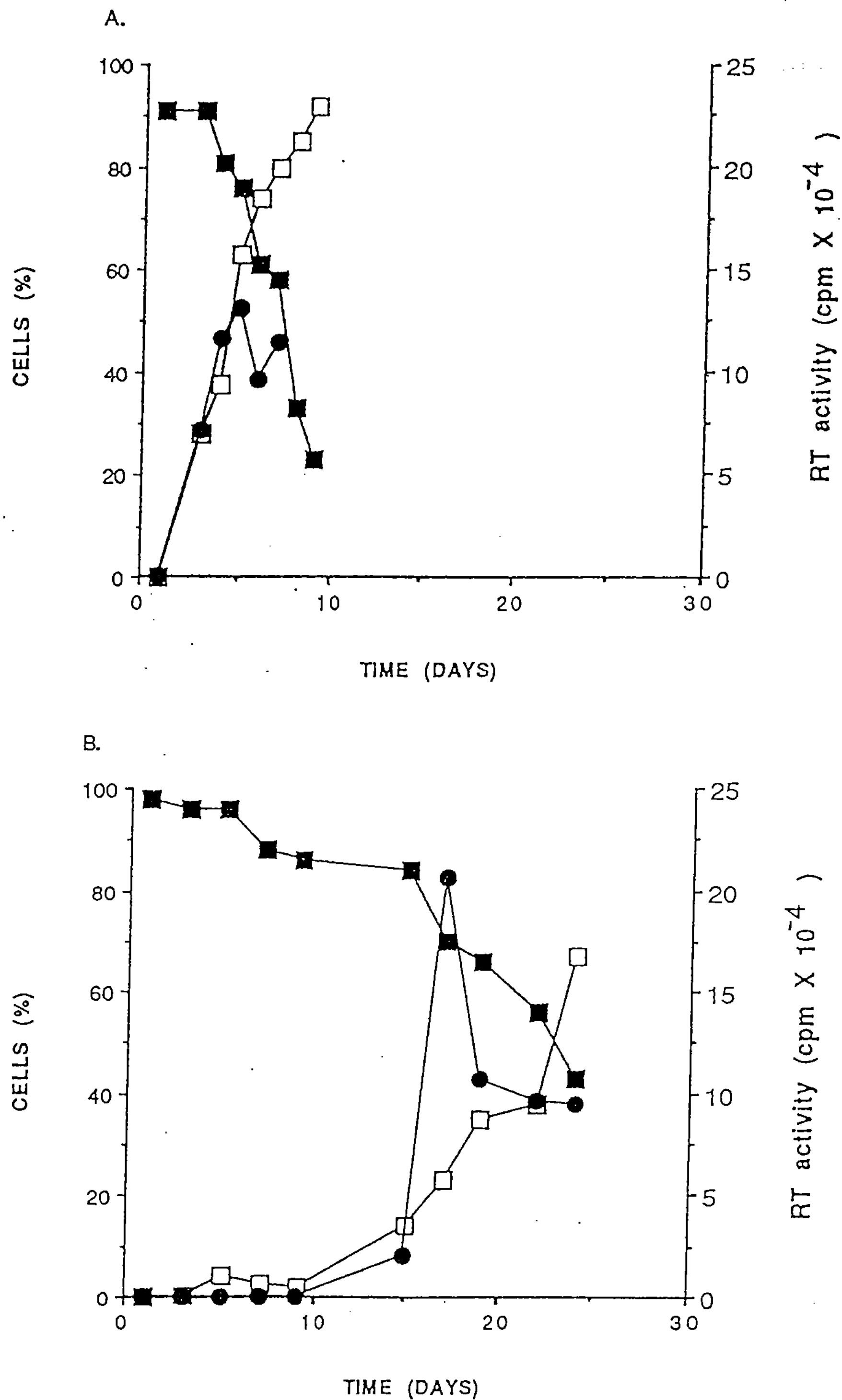

FIGURE II-2. **Viral antigen expression and reverse transcriptase activity.** H9 (A) or HL-60 (B) cells were infected with HIV NL4-3 as described in the legend to Figure II-1. The percentage of cells that reacted with a pooled patient serum (-□-), the viability of the culture (-■-) and the RT activity in the culture medium (-●-) are plotted.

Table II-1. CD4 Expression and Blocking on HL-60 and H9 Cell Lines.

CELL LINE	CD4+ (%)[a]	HIV[b]	Leu3a[c]	IFA-positive (%)[d]	
				Day 3	Day 24
H9	96	-	-	n	n
		+	-	19	82
		+	+	n	n
HL-60	85	-	-	n	n
		+	-	5	35
		+	+	n	n

[a] Percentage of the culture which was CD4-positive as determined by FACS analysis using the monoclonal antibody OKT4.

[b] HIV NL4-3 was used to infect cells at an MOI of 0.5 TCID/cell. -, Mock infected; +, HIV-infected.

[c] Cells were pre-incubated with Leu3a (240 ng/ml) 10 min. prior to addition of virus. -, No Leu3a added; +, Leu3a added. After 24 hrs cultures were pelleted, washed, and suspended in medium without Leu3a.

[d] Percentage of IFA-positive cells determined by IFA staining using an HIV positive pooled patient serum as the primary antibody. The n represents <1 cell per 1000 cells scored positive.

Table II-2. Granulocytic Differentiation of HIV Infected HL60 Cells

CELL LINE	DMF[a]	PERCENT IFA+[b]	PROMYELOCYTE (%)[c]	MYELOCYTE (%)[c]	PMN (%)[c]	SUPEROXIDE (O_2^-)[d]
HL-60	-	0	98	2	0	
	+	0	0	0	90	84
PLB985	-	0	97	3	0	
	+	0	0	18	82	ND[f]
PERSISTENTLY INFECTED LINES[e]						
HL-60 IIIB	-	19	97	3	0	
	+	21	0	12	88	158
HL-60 NL4-3	-	14	96	4	0	
	+	19	0	13	87	251
HL-60 PM213	-	59	98	2	0	
	+	55	0	10	90	141
PLB985 IIIB	-	15	98	2	0	
	+	16	0	18	82	ND
CLONAL LINES[g]						
HL-60 C1	-	0	98	2	0	
	+	0	0	13	87	ND
HL-60 IIIB-1	-	33	97	3	0	
	+	36	0	9	91	84
HL-60 IIIB-2	-	19	99	1	0	
	+	24	0	20	80	106

[a] Cells scored 6 days after treatment with dimethyl formamide (DMF); "-" = untreated cells, "+"= 80mM treated.

[b] The percentage of indirect immunofluorescence (IFA) positive cells using an HIV-1 positive pooled patient serum as the primary antibody.

[c] Percentage of cells of each stage of differentiation identified by morphological observation after Wright-Giemsa staining as described in materials and methods. The numbers represent an average of three determinations of >100 cells scored in two separate induction experiments.

[d] NBT reduction assay performed as described in materials and methods; "-" = no superoxide produced, "+"= superoxide produced of $>0.08 A_{515nm}/15min/10^6 cells$.

[e] Cultures 36 days post infection.

[f] ND = not determined.

[g] Expansion of single cell clones of either uninfected, HL-60 (C1) or persistently infected HL-60 IIIB cell lines as described in materials and methods.

CHAPTER III

REPLICATION OF THE HIV-1 T-LYMPHOTROPIC ISOLATE, NL4-3, IN HL-60 CELLS IS RESTRICTED AT SEVERAL STEPS IN THE VIRAL LIFE CYCLE

INTRODUCTION

A complex population of highly related but distinct HIV-1 isolates is found in HIV-1 infected individuals (21, 151-158). These have been termed quasispecies (157, 161). The many isolates that replicate in the patient simultaneously might have very different biological phenotypes and the separate but coordinated effect of these multiple quasispecies may contribute to a complex disease such as AIDS. The ability of the quasispecies to mutate during the course of disease could have important implications for pathogenesis. Therefore, it is important to understand the cellular pressures that control the changing composition of quasispecies.

Several mechanisms of generating viral diversity have been described (191). One mechanism of generating changes involves misincorporation of nucleotides due to the viral RNA polymerase, Reverse Transcriptase (RT), which has a high error rate and lacks proofreading ability (181), and/or to the cellular RNA polymerase II which also has no known proofreading ability (309). Another mechanism of generating viral genomic changes involves major substitutions in viruses that are introduced by viral recombination events that occur between two viral genomes or between viral and cellular sequences (187, 188). The cellular pressures that influence these changes are poorly understood.

As a first step in defining these pressures, various stages of the viral life cycle of HIV NL4-3 were examined in the human promyelocytic cell line, HL-60 compared to the life cycle of the virus in the human T cell line, H9. I previously reported that although HIV-1 infection of HL-60 cells did not alter cellular differentiation, the virus did productively infect and was cytopathic to HL-60 cells (Chapter II). The life cycle of HIV-1 NL4-3 was restricted in myeloid cells compared to T cells (Fig. II-2). In the current studies, I report that NL4-3 stably adapts when grown in HL-60 cells resulting in a virus which is more rapidly cytopathic to HL-60 cells. This viral adaptation overcomes restrictions in viral replication which occur at more than one step in the viral life cycle.

MATERIALS AND METHODS

Cell lines, viruses and infections. The human promyelocytic cell lines, HL-60 (Chapter II), the human lymphoblastoid cell line, H9 (Chapter II), the human promyelocytic cell line PLB985 (Chapter II, 251), and an HTLV-I immortalized cell line (244), C8166, (a gift of Dr. H. Robinson, Univ. Mass. Med. Ctr.) were maintained at densities between 2×10^5 and 2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone). Cell counts and viabilities were performed using a hemacytometer and trypan blue dye exclusion.

The HIV-1 strains used were the cloned viral isolate NL4-3 (104) and NL4-3(M) harvested from infected HL-60 cells. The infectious molecular clone pNL4-3 is a recombinant proviral clone that contains DNA from the HIV-1 isolates NY5 and LAV (104). NL4-3 virus was prepared by transfecting pNL4-3 DNA into H9 cells. Briefly, H9 cells (5×10^6) were incubated in 0.025% trypsin-EDTA/RPMI 1640 buffered with 0.1M Tris-HCl (pH7.5) at room temperature for 4 minutes. The trypsinized cells were incubated with 5-10μg of pNL4-3 in RPMI 1640 buffered with Tris-HCl (pH 7.5) and 200 μg/ml of DEAE-dextran for twenty minutes at room temperature. The transfected cells were diluted in culture medium, washed, and grown in RPMI 1640 supplemented with 15% fetal bovine serum. When greater than 10% of the transfected cultures expressed viral antigens, cell-free supernatants were collected at forty-eight hour intervals. Infectious viral titers were determined by infecting C8166 cells with serial dilutions of virus stocks. Twenty-four hours after infection the number of infected cells, or cells that react with an HIV-1 positive pooled patient serum, was determined by an indirect immunofluorescence assay (IFA) as previously described (Chapter II). All titers are expressed as C8166 tissue culture infectious dose per cell (TCID/cell). Virus used in PCR

experiments was DNase treated in RPMI 1640 containing 10mM $MgCl_2$ and 20μg/ml RQ DNase I (Promega) at 37°C for thirty minutes (23). All virus was stored at -80°C.

H9 and HL-60 cells were subcultured and infected the next day in the presence of 2μg/ml polybrene at the multiplicity of infection (MOI) specified in the Figure legends. Twenty-four hours later, the cells were pelleted, washed with PBS, and maintained at 1 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum. The viabilities of the cultures were monitored by trypan blue dye exclusion as indicated. Cell-free virus was obtained from chronically infected H9 or HL-60 cells 30 days after the original infection. Virus collected from HL-60 cells is designated NL4-3(M).

Northern hybridization analysis. Total cellular RNA from uninfected and NL4-3 or NL4-3(M) infected cell lines was extracted by the guanidinium hydrochloride procedure (257). Ten μg of each RNA sample and 10μg of an RNA ladder (BRL), used as a size standard, were denatured with formaldehyde-formamide and separated by electrophoresis in 1.0% agarose gels in the presence of 6.7% formaldehyde. The RNA was transferred overnight in 20X SSC (3M sodium chloride, 0.3M sodium citrate) onto nylon membrane (Zetabind, CUNO). The membranes were baked for 30 minutes at 80°C, pre-hybridized at 42°C overnight in 50% formamide, 5X SSPE (0.75M NaCl, 40mM NaH_2PO_4, 4mM EDTA), 5X Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% sodium dodecyl sulfate (SDS), and 100μg/ml salmon sperm DNA, and hybridized at 42°C overnight by adding 10^7 cpm of (^{32}P)-labeled DNA from an 8.0kb Ava I fragment of pNL4-3 DNA in 50% formamide, 5X SSPE, 0.1% SDS 2X Denhardt's, 0.6% sodium dextran sulphate. After hybridization, membranes were washed at room temperature for fifteen minutes in 2X SSC/0.1% SDS, then three times (15 minutes each) at 42°C in 0.1X SSC/0.1% SDS and subjected to autoradiography.

Kinetics of entry. The kinetics of entry of NL4-3 into H9 or HL-60 cells and NL4-3(M) into HL-60 cells was determined using the procedure described by Srivastava and co-workers (250). Briefly, H9 and HL-60 cells (5 x 10^5 cells per well) were

incubated with NL4-3 or NL4-3(M) (MOI of >0.4 TCID/cell) and 2μg/ml polybrene. At timed intervals after infection, Leu3a was added to the culture to a final concentration of 240ng/ml. Virus was pre-incubated with 240ng/ml of Leu3a prior to addition to H9 or HL-60 cells for the zero time point. Twenty-four hours after infection, the cultures were washed with PBS and suspended at 1×10^6 cells/ml in RPMI 1640/10% fetal bovine serum supplemented with 240ng/ml Leu3a and 2μM ddC to ensure single cycle infection. Slides were prepared for IFA 60 and 72 hours after infection.

Polymerase chain reaction (PCR) analysis. H9 and HL-60 cultures were infected with NL4-3 at an MOI of >0.20 TCID/cell. At timed intervals after infection 2×10^6 cells were pelleted by centrifugation, washed in PBS, and suspended at 6×10^6 cells/ml in PCR lysis buffer (50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20, and 6ng/ml Proteinase K). Samples were incubated at 56°C for one to two hours, heated to 100°C for ten minutes, and stored at -20°C. The pNL4-3 specific primer pair used: R1, 5'-GGCTAACTAGGGAACCCACTGCTTAA-3' (nt 496 to nt 518) and NC1, 5'-CCGAGTCCTGCGTCGAGAGATC-3' (nt 680 to nt 701). This primer pair is predicted to amplify a 206 bp fragment. Similar primers have been used by other investigators to measure newly synthesized DNA (23). Beta-globin primers B1, 5'-CAACTTCATCCACGTTCACC-3' (nt -73 to nt -54) and B2, 5'-GAAGAGCCAAGGACAGGTAC-3' (nt -195 to nt -176) which are predicted to amplify a 268 bp fragment were used as internal amplification standards. PCR reactions were performed using 1.0μM concentration of each primer in a 50 μl reaction containing 200μM concentration of each of the four deoxynucleoside triphosphates, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 1.25 Units of *AmpliTaq* DNA Polymerase (Perkin Elmer Cetus), and cell lysate of 6×10^4 cells. The reactions were overlaid with a drop of mineral oil and subjected to 35 cycles (denaturation for 45 seconds

at 95°C, annealing for two minutes at 55°C, extension for three minutes at 72°C, and a final ten minute extension at the 35th cycle) in a Thermolyne thermocycler.

8E5 cells which contain one provirus per cells (258) were used to quantitate the amount of newly reverse transcribed DNA in cell lysates from NL4-3(M) infected HL-60 cells and NL4-3 infected H9 or HL-60 cells collected 6 hours after infection. All cell lysates were serially diluted with cell lysate from uninfected HL-60 cells, such that the cell number remained 6 x 10^6 cells/ml. Ten µl (6 x 10^4 cells) of each cell lysate were subjected to PCR amplification as described above using primer pair R1/NC1. PCR products were separated by electrophoresis through 2.0% agarose gels prior to Southern blot analysis described below. In separate experiments the HIV-1 specific primer pair pol 1/pol 2 is used for amplification and quantitation of reverse transcribed NL4-3 DNA (Chapter IV).

High molecular weight DNA. Genomic DNA was prepared by standard techniques (259) for the isolation of high molecular weight DNA free of unintegrated, episomal DNA. Briefly, HIV-infected H9 and HL-60 cultures or mock infected cultures were pelleted by centrifugation (500g for 5 minutes), washed twice with PBS, and suspended at 1 X 10^7 cells/ml in digestion buffer (100mM NaCl, 10mM Tris-HCl pH 8.0, 25mM EDTA, 0.5% sodium dodecyl sulfate, 0.1mg/ml proteinase K). The samples were incubated at 55°C for 12 to 18 hours and then thoroughly extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The aqueous layer was transferred to a new tube and 1/2 the volume of 7.5M ammonium acetate and 2 volumes of 100% ethanol was added. The DNA was recovered by centrifugation at 3000g for 30 minutes. The DNA was rinsed twice with 70% ethanol, air dried, and suspended in 10mM Tris-HCl pH 8.0/ 1mM EDTA. Each sample (20 µg) was digested with Xho I which cuts once in the viral genome (nt 8887) and electrophoresed in 0.7% agarose gels for Southern blot analysis described below.

Southern hybridization analysis. After electrophoresis, all gels were treated for 10 minutes with 0.25N HCl and the DNA was transferred to nylon membranes (Zetabind, CUNO) in 0.4N NaOH for 5 to 18 hours. Following pre-hybridization for 2 hours at 65°C in 4X SSCP (0.5M sodium chloride, 60mM sodium citrate, 60mM sodium phosphate), 1 X Denhardt's (0.1% Ficoll 400, 0.1% polyvinylprrolidone, 0.1% bovine serum albumin), 0.5% sodium dodecyl sulfate, and 250ug/ml salmon sperm DNA, the membranes were hybridized overnight at 65°C in buffer containing 4X SSCP, 1X Denhardt's, 1.0% sodium dodecyl sulfate, 10% sodium dextran sulphate, and 10^7 cpm of ^{32}P-labeled probe. The probe was prepared by random prime labeling (Random Primers labeling kit, Bethesda Research Laboratories) an 8.0Kb Ava I DNA fragment of pNL4-3. Following hybridization, membranes were washed in 3X SSCP, 4X Denhardt's, 0.1% SDS briefly at room temperature, in 3X SSCP/0.1% SDS for fifteen minutes at 65°C, in 1X SSCP/0.1% SDS for fifteen minutes at 65°C, and in 0.1X SSCP/0.1% SDS for 15 minutes at 65°C and then subjected to autoradiography. Densitometric analysis was performed on autoradiographs using a CS-9000 Dual-wavelength Flying-spot Scanner (Shimadzu Co., Kyoto, Japan).

RESULTS

Viral adaptation. As previously reported, HIV-1 infection of HL-60 cells initially results in expression of viral proteins on only a small percentage of cells and in a low level of cell-free reverse transcriptase activity. Fifteen or more days after HIV-1 infection of HL-60 cells, a decrease in viability of the culture with a concomitant increase in the percentage of cells expressing viral proteins to levels equivalent to HIV-1 infected H9 cells was observed (Chapter II, Fig. II-2).

I hypothesized that during the restricted replication of NL4-3 in HL-60 cells, a viral variant was produced by random mutation that has adapted to efficient replication in HL-60 cells and was highly cytopathic for HL-60 cells. To test this hypothesis, virus was collected 30 days after infection from NL4-3 infected HL-60 cells and used to infect both H9 and HL-60 cells. In parallel, NL4-3 harvested 7 days after infection of H9 cells was used to infect both H9 and HL-60 cells. The viabilities of the cultures were monitored for 30 days by vital dye exclusion (Fig. III-1). NL4-3 harvested from H9 cells and NL4-3(M) harvested from HL-60 cells had similar cytopathic effects on H9 cells. Surprisingly, these viruses had strikingly different cytopathic effects on HL-60 cells. NL4-3 showed delayed cell death on HL-60 cells (Fig. III-1B) with a mean survival time (MS_{50}) of 22 days, while NL4-3(M) rapidly killed HL-60 cells (MS_{50}=8 days). These data suggest that a viral variant is produced in HL-60 cells and this variant is cytopathic to HL-60 cells.

To test whether this observation was specific for NL4-3, the experiment was repeated using another cloned HIV-1 isolate, PM213 (Chapter II). The results were indistinguishable from those shown in Figure III-1 (data not shown). In addition, I previously demonstrated that the cytopathic phenotype of the HIV-1 isolate IIIB is identical to that of PM213 and NL4-3 in H9 and HL-60 cells (Chapter II, Fig. II-1). Therefore, the viral adaptation was not unique to NL4-3 and probably reflects a biological process of viral adaptation or selection of a cytopathic HIV-1 variant in HL-60 cells.

To determine if a virus with this cytopathic phenotype could be generated by multiple rounds of viral replication in another cell line, H9 cells were infected with NL4-3 and virus was harvested from the survivor cell population 30 days after infection. This virus was used to infect both H9 and HL-60 cells. The results were compared to the results obtained in a similar experiment using virus harvested from H9 cells 7 days after infection. Figure III-2 shows that the kinetics of cell death in HL-60 cells was similar for NL4-3 harvested from H9 cells 7 or 30 days after infection. These results demonstrate that NL4-3 has a delayed cytopathic effect on HL-60 cells regardless of the number of rounds of replication it undergoes in H9 cells. In addition, these results are consistent with the suggestion that there is a selection for viral adaptation that is specific to HL-60 cells rather than a selection based on the number of rounds of viral replication.

To determine if the cytopathic phenotype of the HL-60 adapted variant, NL4-3(M), was stable, the virus was propagated through two sequential rounds of infection of H9 cells. Virus harvested after the second passage in H9 cells was used to infect both H9 and HL-60 cells and the viability of the cultures monitored. As shown in Figure III-3, passage of NL4-3(M) through H9 cells did not alter the virus' biological phenotype. These results indicate that the change in the biological phenotype is stable and that it is not selected against by growth in H9 cells. In addition, these results rule out the possibility that the increased cytopathic effect of NL4-3(M) was due to cytokines or other factors produced by and carried over from HL-60 cells.

To determine if the increased cytopathic phenotype of NL4-3(M) was specific only for HL-60 cells, another myeloid leukemia cell line, PLB985, was infected with NL4-3(M) and NL4-3. The results indicate that NL4-3(M) had a more rapid cytopathic effect on PLB985 cells than NL4-3 (Figure III-4). Therefore the cytopathic phenotype of NL4-3(M) is not specific for HL-60 cells but also affects another myeloid cell line.

To determine if the phenotype of NL4-3(M) was representative of the majority of the virus in the population, virus was harvested four days after infection of HL-60 cells with NL4-3(M), titered, and used to infect HL-60 cells at an MOI of 1.0 to 0.01 TCID/cell. The cells were monitored for the rapid cytopathic phenotype of NL4-3(M). The results from these experiments demonstrate that even at an MOI of 0.01 TCID/cell NL4-3(M) rapidly killed HL-60 cells (Fig. III-5A). At the same MOI of 0.01 TCID/cell, NL4-3 kills HL-60 cells more slowly (Fig. III-5B). However, at an MOI of 0.01 TCID/cell, NL4-3 still kills H9 cells rapidly (Fig. III-5B). This indicates that the cytopathic phenotype of NL4-3(M) is the predominant phenotype of the viruses in the population.

Kinetics of viral entry. To determine if the delayed cytopathic effect of NL4-3 on HL-60 cells was due to a delay in the timing of entry of the virus into HL-60 cells, NL4-3 was used to infect both H9 and HL-60 cells and compared to NL4-3(M) infected HL-60 cells. Leu3a, a monoclonal antibody to CD4, was added at timed intervals after infection to block viral entry. This method has been used by other investigators to determine the entry rates of HIV-1 into various cell lines (250). As previously reported, addition of Leu3a prior to the addition of virus completely blocked HIV-1 infection of both cell lines (Chapter II, Table II-1). In addition to Leu3a, ddC (2μM) was added to the culture to prevent reverse transcription and viral spread. In the presence of these two inhibitors, only virus that enters the cell and is reverse transcribed before the addition of Leu3a and ddC score positively for viral surface antigen expression by IFA. To determine the maximum number of virus expressing cells, two time points, 60 hours and 72 hours post infection, were scored and an average of the two time points were plotted.

The kinetics of NL4-3(M) entry into HL-60 cells and NL4-3 entry into HL-60 and H9 cells is shown in Figure III-6. The maximum percentage of virus expressing cells in NL4-3 infected H9 cultures (14%) was 3.5 or 2.3 fold higher than in NL4-3 infected HL-60 cultures (4%) or NL4-3(M) infected HL-60 cultures (6%), respectively. Nevertheless,

the entry time, defined as the time point of Leu3a addition at which 50% of the maximum IFA positive cells are observed, was determined to be between 4 and 4.5 hours for NL4-3 in H9 and HL-60 cells and 4 hours for NL4-3(M) into HL-60 cells. This time for viral entry into H9 cells is consistent with that previously reported by Srivastava and co-workers (250). Therefore differences in the timing of entry cannot explain the 10 to 15 day delay in the life cycle of the virus in HL-60 cells.

Reverse transcription. Once the virus has entered the cell, its RNA genome is reverse transcribed into DNA. A PCR analysis using primers R1 and NC1 to assay for the presence of newly reverse transcribed DNA in NL4-3 infected H9 and HL-60 cells was performed. The R1/NC1 primer pair amplifies a 206 bp fragment at the R/5' non-coding sequence junction. The non-coding region is 5' to the primer binding site and thus the products of this amplification represent newly reverse transcribed DNA. Beta-globin specific primers B1 and B2 which amplify a 268 bp fragment were used to detect cellular DNA. It has been shown that DNase treatment of viral particles eliminates nonspecific DNA sequences carried on the surface of virions (23, 260). Therefore, NL4-3 virions were treated with DNase as described in Materials and Methods. Figure III-7 shows a PCR analysis of untreated and DNase treated stocks. DNase treatment completely eliminated the beta-globin DNA from the virus stock and greatly decreased the amount of viral DNA (lanes 2, Fig. III-7A and B) compared to untreated stocks (lanes 3, III-7A and 7B). The presence of viral DNA in DNase treated stocks is consistent with the observation that partial reverse transcription occurs in the virion prior to entry into a cell (23, 260).

Cell lysates were prepared at timed intervals after infection. To be sure I was measuring newly reverse transcribed DNA, HL-60 cells were pre-treated for 15 minutes with 2μM ddC before addition of DNase treated NL4-3. Cell lysates were prepared 2 hours after ddC treatment. These lysates were then subjected to PCR amplification using beta-globin specific primer pair, B1/B2 to control for the amount of DNA analyzed (Fig.

III-8B). These same lysates were PCR amplified and the products analyzed for the presence of viral DNA using primer pair R1/NC1. Two hours after infection viral DNA was detected in both infected H9 and HL-60 cells but not in ddC treated HL-60 cells (Fig. III-8A). In infected H9 cells, a high level of newly reverse transcribed DNA was detected continuously over five days, consistent with a spreading infection in H9 cells (Fig. III-8A). The results are different in HL-60 cells where newly reverse transcribed DNA decreased with time. These data support the observation of Butera and co-workers (230) that HIV-1 exists as extrachromosomal viral DNA in infected HL-60 cells and is not faithfully transmitted during cell division. These data are also consistent with a restriction in the spread of the virus in the HL-60 culture.

To determine if there was a quantitative difference in the amount of newly reverse transcribed DNA in infected H9 and HL-60 cells, lysates from cells 6 hours after NL4-3 or NL4-3(M) infection were diluted serially and amplified using an HIV-1 specific primer pair (Fig. III-8C). For quantitation, amplification of cell lysates from serially diluted 8E5 cells, which contain a single provirus, was used to generate a standard curve by densitometric analysis of the data from Fig. III-8D. The samples shown in lanes 3, 6, and 9 of Figure III-8C gave densitometric readings that fell in the linear portion of the curve and were used to calculate the number of reverse transcribed DNA copies per sample (Fig. III-8E). There is a 3.6 fold higher amount of reverse transcribed product in NL4-3(M) infected HL-60 cells and NL4-3 infected H9 cells (23.7 copies/1 x 10^3 cells) compared to NL4-3 infected HL-60 cells (6.5 copies/1 x 10^3 cells). These data are consistent with the 3.5 fold difference in the NL4-3 viral antigen expression on the cell surface of H9 and HL-60 cells seen in the entry experiment (14% vs. 4%) shown in Figure III-6. The results of this and three separate experiments using HIV-1 specific primer pairs, R1/NC1 and pol1/pol2, varied by no more than 20% (3.6X, 3.64X, 17.5X, 3.56X) and consistently showed a higher amount of reverse transcribed product in NL4-3 infected H9 cells than HL-60 cells. These results, in conjunction with the result that the timing of entry of NL4-

3 into H9 and HL-60 cells was similar, suggest one restriction in replication of NL4-3 in HL-60 cells occurs at a step before reverse transcription.

If the decrease in the percent of infected cells accounts for the delay of NL4-3 replication in HL-60 cells, I would predict that a reduction in the number of infected, fully permissive H9 cells would give a similar delay in cytopathicity as seen in HL-60 cells. This was tested by comparing the viability of NL4-3 infected H9 cells at an MOI of 0.5 TCID/cell versus an MOI of 0.01 TCID/cell (Fig. III-5B). A 50 fold decrease in the MOI does not significantly change the kinetics of replication of NL4-3 in the fully permissive H9 cells. However, reducing the MOI of NL4-3 to 0.01 TCID/cell caused a further delay in the replication of the virus in HL-60 cells (Fig. III-5B). Thus, the restriction in NL4-3 entry into HL-60 cells alone is not sufficient to explain the delay seen in the spread of the virus in the HL-60 cultures.

NL4-3 recruitment of HL-60 cells. To assay the efficiency of viral spread in HL-60 cells, high molecular weight DNA was isolated from H9 and HL-60 cells 5 days after infection with either NL4-3 or NL4-3(M). The DNA was digested with the restriction endonuclease Xho I which cuts once in the NL4-3 provirus (nt 8887) and was analyzed by Southern blotting (Fig. III-9). Proviral sequences were detected as a smear of HIV specific DNA which migrated as >9.7 kb in both NL4-3 and NL4-3(M) infected H9 cells as well as in NL4-3(M) infected HL-60 cells. In addition, viral specific bands, possibly derived from circular DNA, were detected at approximately 9.7 kb and 0.9 kb. In contrast, no proviral or circular DNA sequences were detected in HL-60 cells infected with NL4-3.

Total RNA was isolated from NL4-3(M) and NL4-3 infected cultures at various times after infection. As expected, three forms of HIV RNA (full length: 9.7 kb, singly spliced: 4.4 kb, and multiply spliced: 2.2 kb) were detected in NL4-3 infected H9 cells and NL4-3(M) infected HL-60 cells 5 days after infection (Fig. III-10A, C). NL4-3 RNA was not detected by Northern blot analysis in infected HL-60 cultures until day 15 after

infection (Fig. III-10B). These data are consistent with restricted replication of NL4-3 in HL-60 cells that results in a low efficiency of viral recruitment of the culture. Furthermore, the variant virus, NL4-3(M) has overcome this restriction and is capable of efficient viral recruitment of HL-60 cells.

DISCUSSION

I have studied the early events in the life cycle of NL4-3 in the human myelomonocytic cell line, HL-60, as a model system for understanding viral adaptation to different cell types. My data as well as that of others show that HIV-1 can infect HL-60 cells (Chapter II, 215, 230, 261, 262). However, in these studies I find that in NL4-3-infected HL-60 cells, a low level of viral protein expression and virus release occurs during the first 15 days after infection (Chapter II). Fifteen days after infection there is a striking increase in viral protein expression, release, and cytopathicity. In this study, I demonstrate that a variant virus, NL4-3(M), can be consistently isolated during the lytic phase of infection of HL-60 cells. NL4-3(M) is more cytopathic to myeloid cells than the parental virus, NL4-3. The phenotype of NL4-3(M) is stable and not due to cytokines or other factors produced by HL-60 cells. Based on the calculation using a viral life cycle of 24 hours and a conservative burst size of 10 particles per infected cell, a highly cytopathic virus in the original population would require only 10 generations to infect the entire HL-60 culture. However, NL4-3(M) takes at least 15 generations to emerge from infected HL-60 cultures. Thus, the phenotype observed is not likely due to the outgrowth of an existing variant in the original viral population but rather to the emergence of a new adapted viral variant after growth in HL-60 cells.

Restricted growth of HIV-1 in different cell types can be the result of multiple, additive steps in the viral life cycle including the kinetics and efficiency of entry, intracellular steps in viral replication, and the magnitude of the viral burst. To analyze the first of these steps, the kinetics of NL4-3 entry and reverse transcription into HL-60 cells and H9 cells were examined. The data demonstrate that NL4-3 enters HL-60 and H9 cells with identical kinetics; however, the number of completed reverse transcribed molecules is consistently higher in H9 than in HL-60 cells as determined by densitometric analysis of Southern blots. This suggests that there is a difference in the efficiency of entry of NL4-3

into these two cells lines. These results are similar to those of Kim *et al.* (263). Their data demonstrate that the quantity of linear viral DNA was 5 to 10 fold higher in H9 cells than in the human promonocytic cell line, U937. An alternative method for quantitating the amount of viral DNA detected in the PCR reactions is competitive PCR (311). This method uses a known amount of an HIV-1 control plasmid added to each sample. The control plasmid contains the same viral DNA region as that being amplified in the samples; however, an internal portion has been deleted such that a smaller product is amplified. This method provides an internal control for amplification variability and primer use and is thus more sensitive than the method used in these studies. A larger difference in the amount of newly reverse transcribed DNA in H9 verses HL-60 cells may have been observed using competitive PCR.

In addition to this restriction, the ten to fifteen day delay observed in the spread of NL4-3 in HL-60 cells compared to H9 cells suggests that there is another restriction in the life cycle of NL4-3 in HL-60 cells that affects a step after viral entry, uncoating, and reverse transcription. This restriction may be specific to the stage of differentiation of HL-60 cells since it has been demonstrated that the stage of differentiation influences viral replication (213, 214). A similar restriction of HIV-1 replication in macrophages has been demonstrated to occur at post entry events. Huang and coworkers (264) have demonstrated that lymphotropic HIV-1 (HIV-1 IIIB) efficiently enters and synthesizes viral DNA during infection of macrophages. They further demonstrate that 2-LTR circular viral DNA is present only in macrophages infected with a macrophage tropic virus (HIV-1 ADA), indicating a restriction at later stages of virus replication and implicating DNA migration to the nucleus as a determinant of HIV-1 tropism.

One characteristic feature of HIV-1 is its high degree of genetic variability. HIV-1 variants show distinct biological features including replication kinetics, serum neutralization, and cytopathic effect (21, 112, 169-175). The development of a highly cytopathic variant virus seen in this system mimics some of the events observed *in vivo.*

Recent studies by Mori *et al.* (265) demonstrate that when SIVmac239 is injected into rhesus monkeys a variant, SIVmac316, arises which replicates more efficiently in macrophages than SIVmac239. I note that the phenotype of this virus is very similar to NL4-3(M). Specifically, the investigators have quantitated the amount of newly synthesized viral DNA 14-16 hours after infection of alveolar macrophages and found that cells infected with a SIVmac239 recombinant containing the envelope gene of SIVmac316, SIVmac239/316ENV, have a three fold higher amount of newly synthesized viral DNA than cells infected with SIVmac239. In addition, since viral DNA increased dramatically between 14 and 66 hours after infection of alveolar macrophages only with SIVmac239/316ENV and not with SIVmac239, they conclude that the restricted replication of SIVmac239 in macrophages is due to a step in the viral life cycle after entry.

My results indicate that the restriction of NL4-3 in HL-60 cells is due not only to a reduction in the efficiency of viral entry into HL-60 cells, but also a restriction at one or more steps in the viral life cycle after reverse transcription. Thus, I suggest that the restricted phenotype of NL4-3 in myeloid cells is controlled by more than one viral gene. I also note that the viral variant, NL4-3(M), produced during HL-60 cell infection, overcomes all restrictions to viral replication in HL-60 cells.

Consistent with these findings are recent reports which suggest that multiple additional viral genes including *vif*, *nef*, *vpu*, and *gag* influence tropism and cytopathicity (103, 107, 110, 138, 160, 162-166, 168, 181, 266). Two candidate genes involved in the restricted growth of NL4-3 in HL-60 cells are *vif* and *vpr*. It has been suggested that Vif protein which is produced at a late step of the viral life cycle increases the infectivity of progeny virus during its production (107, 110), perhaps as much as 100 to 1000-fold (100). Vif is also involved in cell-to-cell transmission (103) and has been implicated in efficient viral DNA synthesis (108, 160). Vpr may also play a role in the replication phenotype observed. Vpr protein is virion associated and is predicted to act either early in the viral life cycle or late in particle assembly and maturation (123). These regulatory

proteins either alone or in combination with other viral and cellular proteins are possible candidates for the restriction in viral spread observed in NL4-3 infection of HL-60 cells.

FIGURES

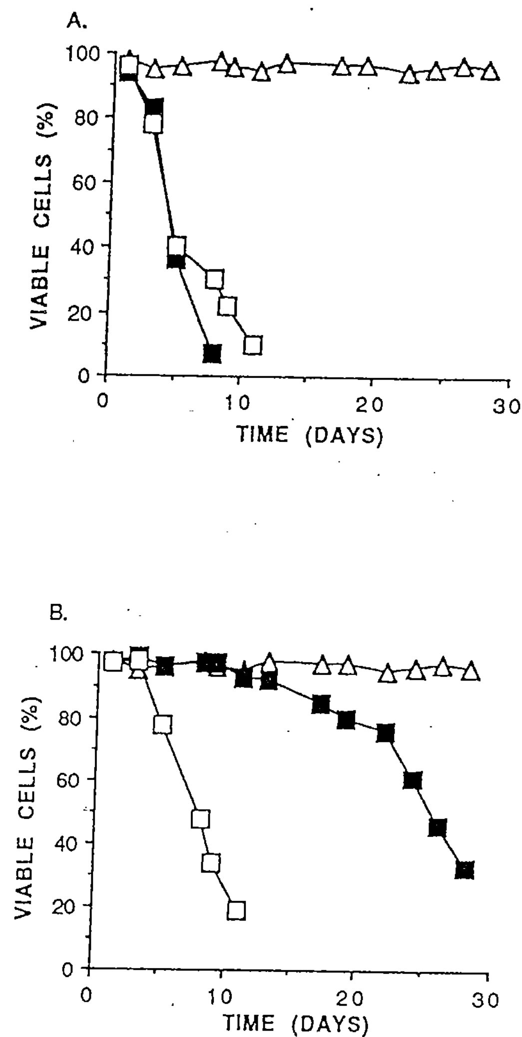

Figure III-1: Viral adaptation. Cell-free virus was harvested from NL4-3 infected HL-60 cells to determine if the virus produced during the lytic phase of infection had a different biological phenotype than NL4-3 harvested from H9 cells. H9 (A) and HL-60 (B) cells were infected as described in Materials and Methods with either NL4-3 harvested from infected H9 cells, (-■-) or NL4-3(M) harvested from infected HL-60 cells (-□-) at an MOI of 0.2 TCID/cell. The open triangle represents mock infected cultures.

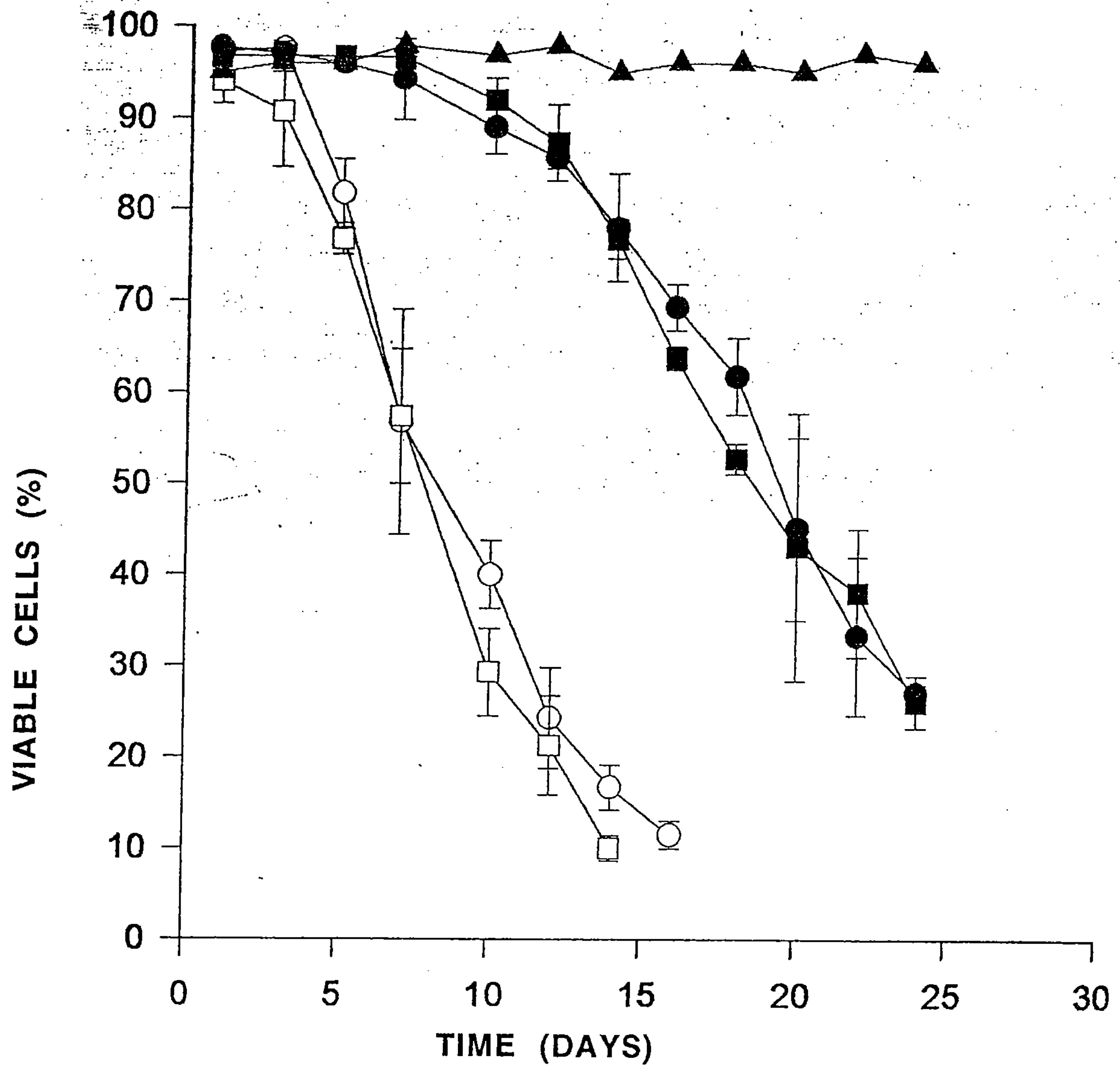

Figure III-2: Cytopathic effect of NL4-3 on H9 and HL-60 cells. Cell-free virus was harvested from NL4-3 infected H9 cells at day seven (---□---) and day thirty (-O-) after infection. These viruses were used to infect H9 (-open symbols-) and HL-60 (-filled symbols-) cells at a multiplicity of infection of 0.25 TCID/cell. The viabilities of the cultures were monitored by vital dye exclusion. The means and standard deviations (error bars) from three independent experiments are plotted in comparison to mock infected HL-60 cells (-Δ-).

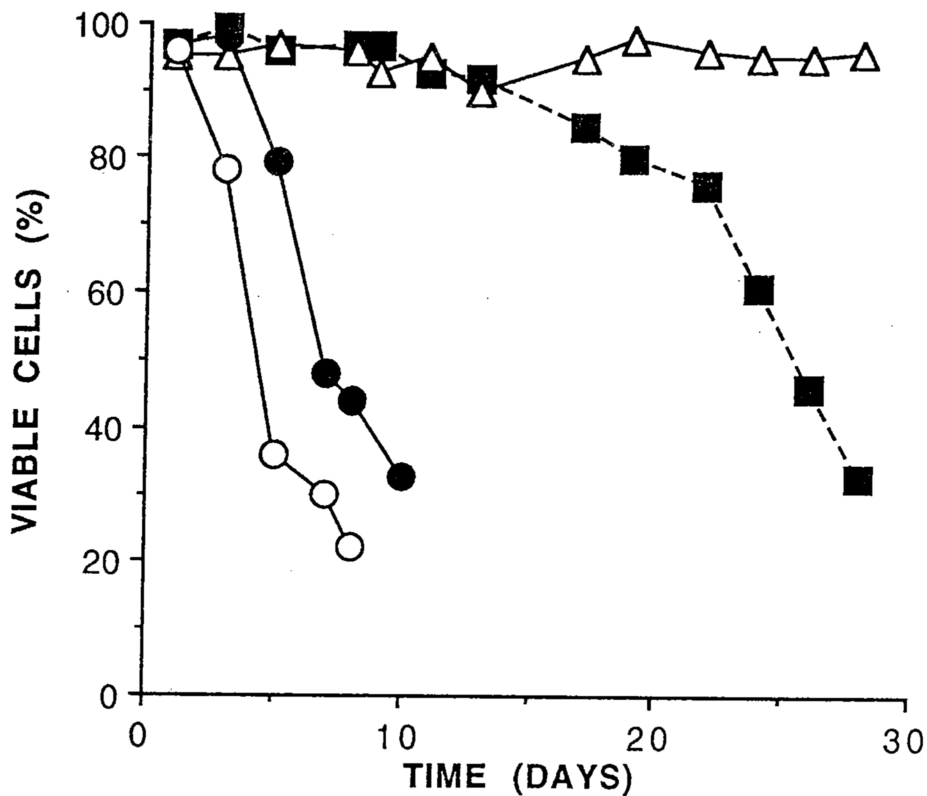

Figure III-3: Stable phenotypic change of HL-60 produced HIV-1. Cell-free NL4-3(M) was harvested from infected HL-60 cells and used to infect H9 cells. Cell-free virus was harvested from NL4-3(M) infected H9 cells and used to infect a new culture of H9 cells. Cell-free virus harvested from the second infection of H9 cells was used to infect logarithmically dividing cultures of H9 (-O-) and HL-60 cells (-●-) at an MOI of 0.2 TCID/cell. The viabilities of the culture were monitored over 30 days by trypan blue dye exclusion and compared to infection of HL-60 cells with NL4-3 (---■---) and control, mock infected cultures (-Δ-).

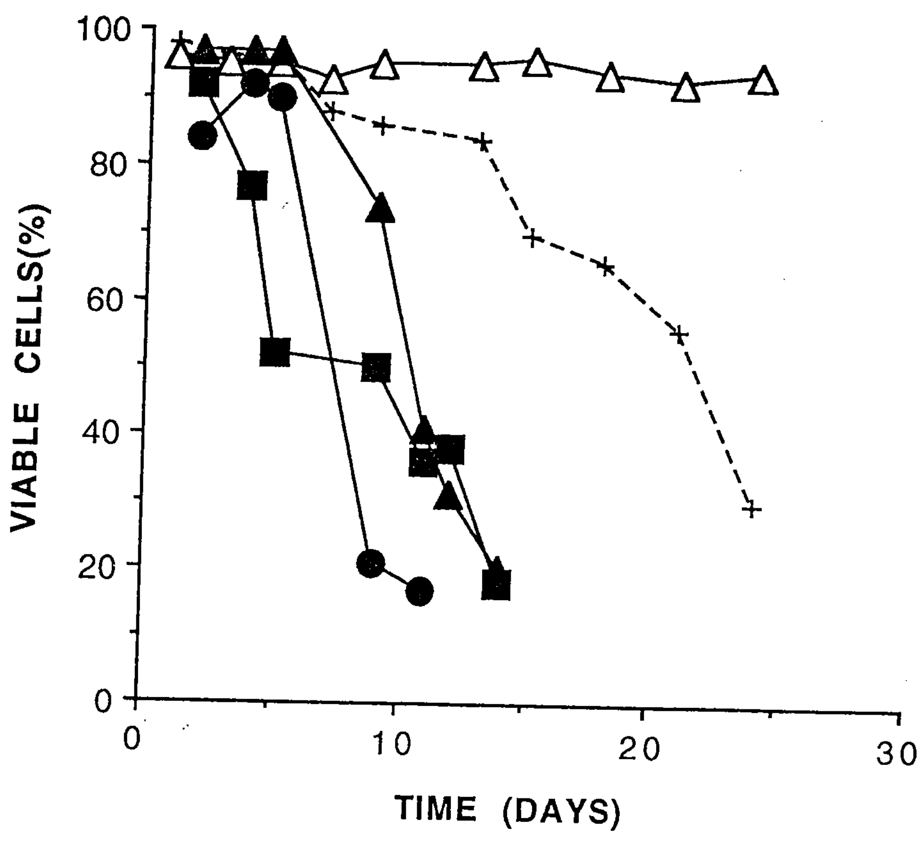

Figure III-4: Virus produced by HL-60 cells is cytopathic to other myeloid cells. Logarithmically dividing HL-60 (-■-), H9 (-●-), and PLB985 (-Δ-) cells were infected at an MOI of 0.2 TCID/cell with NL4-3(M). The viabilities of the culture were compared to infection of PLB985 with NL4-3 (- + -) and control, mock infected cultures(-Δ-).

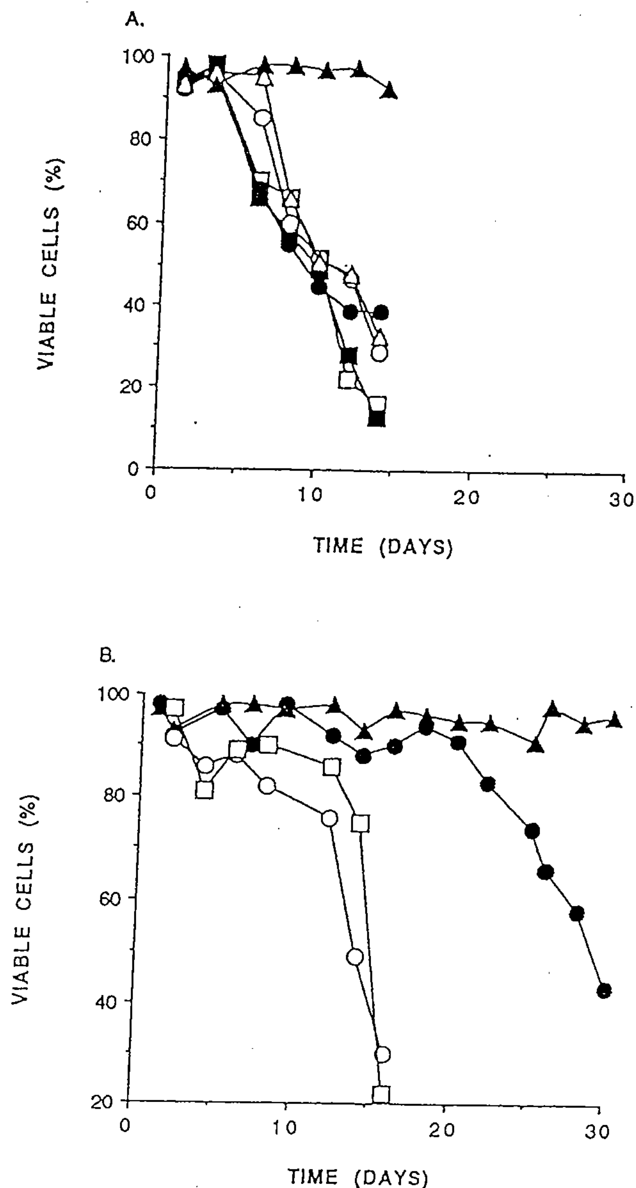

Figure III-5: Representation of adapted virus in NL4-3(M) population. (A) Logarithmically dividing HL-60 cells were either mock infected (-Δ-) or infected with varying MOI's of NL4-3(M) (MOI=1 TCID/cell -□-, MOI=0.5 TCID/cell -■-, MOI=0.1 TCID/cell -●-, and MOI=0.01 TCID/cell -O-). In addition, logarithmically dividing H9 cells (-Δ-) were infected with NL4-3(M) at a MOI of 0.01 TCID/cell. The viabilities of the cultures were monitored over 30 days by trypan blue dye exclusion. (B) In a parallel experiment, NL4-3 was used to infect H9 cells at an MOI=0.5 TCID/cell (-□-) or H9 cells (-O-) and HL-60 cells (-●-) at an MOI=0.01 TCID/cell. The viabilities of these cultures were monitored over thirty days and compared to mock infected (-Δ-) H9 cells.

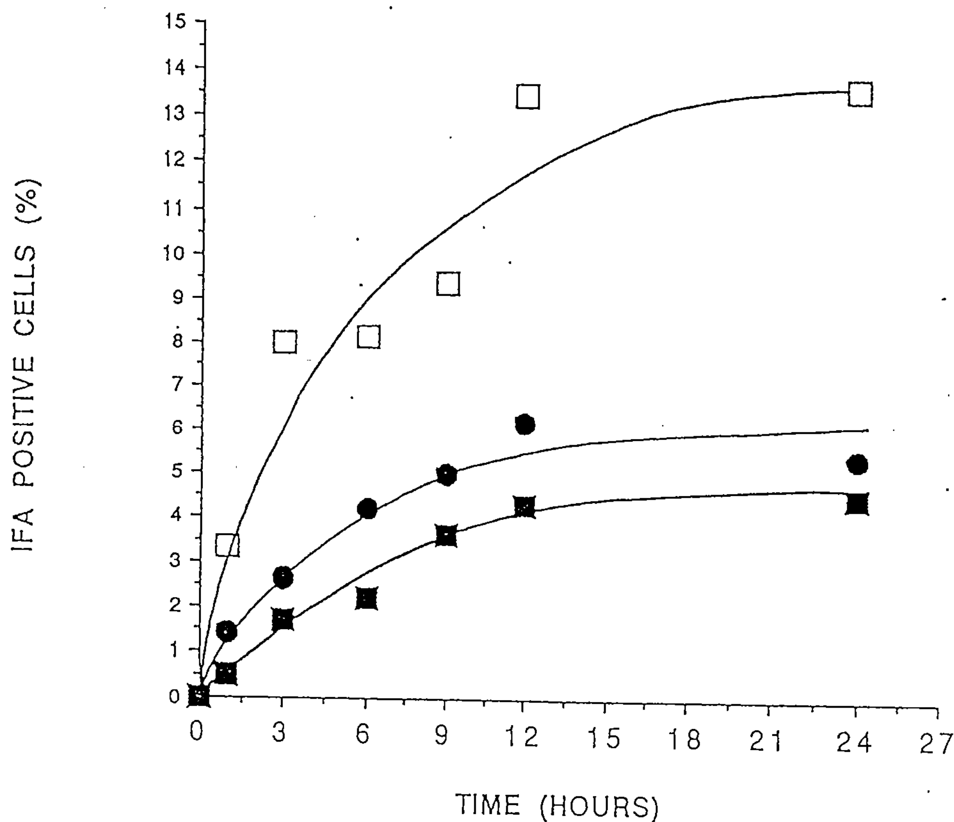

Figure III-6: HIV-1 Entry. To determine if NL4-3 enters H9 and HL-60 cells at a similar rate, a time course of escape from Leu3a block was performed. H9 (-□-) or HL-60 (-■-) cells were infected with NL4-3 at time zero and compared to HL-60 cells infected with NL4-3(M) (-●-) at time zero. At various time points after infection 240ng/ml of Leu3a was added. Twenty-four hours after infection cultures were washed with PBS and suspended in media plus 240ng/ml Leu3a and 2μM ddC to ensure single cycle infection. IFA was performed on each culture at 60 and 72 hours after infection to ensure that all cells were given sufficient time to express viral surface antigen. The results are plotted as percent positive cells versus time (hours) of Leu3a addition. The entry time was determined as the time at which half of the virus entered the total number of positive cells.

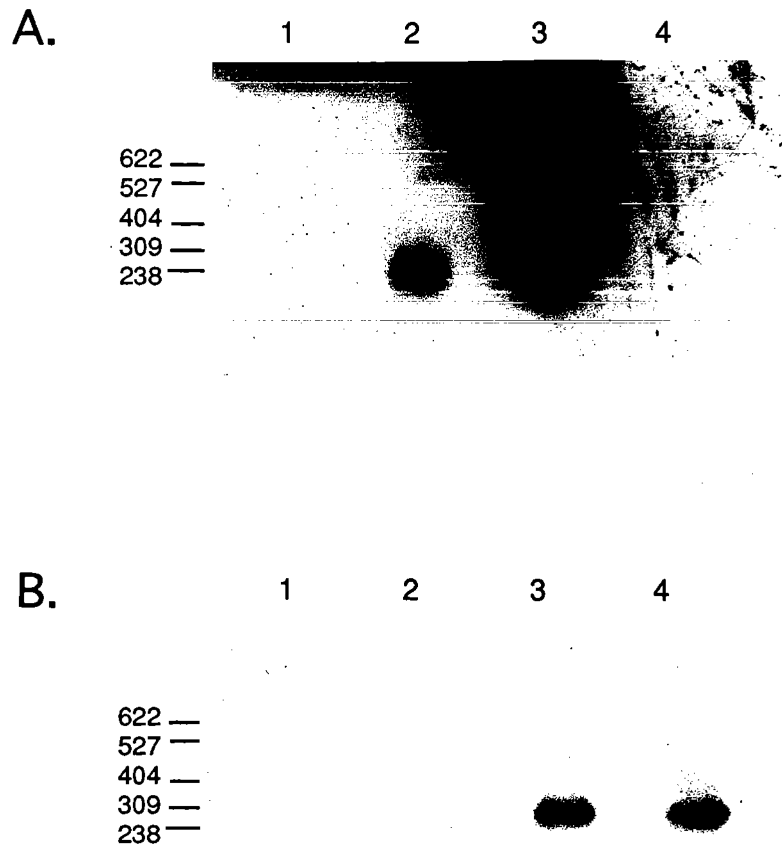

Figure III-7: Detection of proviral DNA in cell-free viral stocks. Cell-free virus was treated with 20 μg/ml DNase for 30 minutes at 37°C. Both DNase treated (lane 2) and untreated NL4-3 virus (lane 3) were PCR amplified using either (A) the primer pairs R1/NC1 or (B) primer pair B1/B2 specific for the beta-globin gene as described in Materials and Methods. The Southern blot in A was exposed to film for 48 hours for detection of viral DNA in the DNase treated virus. The Southern blot in B was exposed to film for 2 hours. Included as controls are a PCR reaction with no template (lane 1) and a lysate of uninfected H9 cells (lane 4).

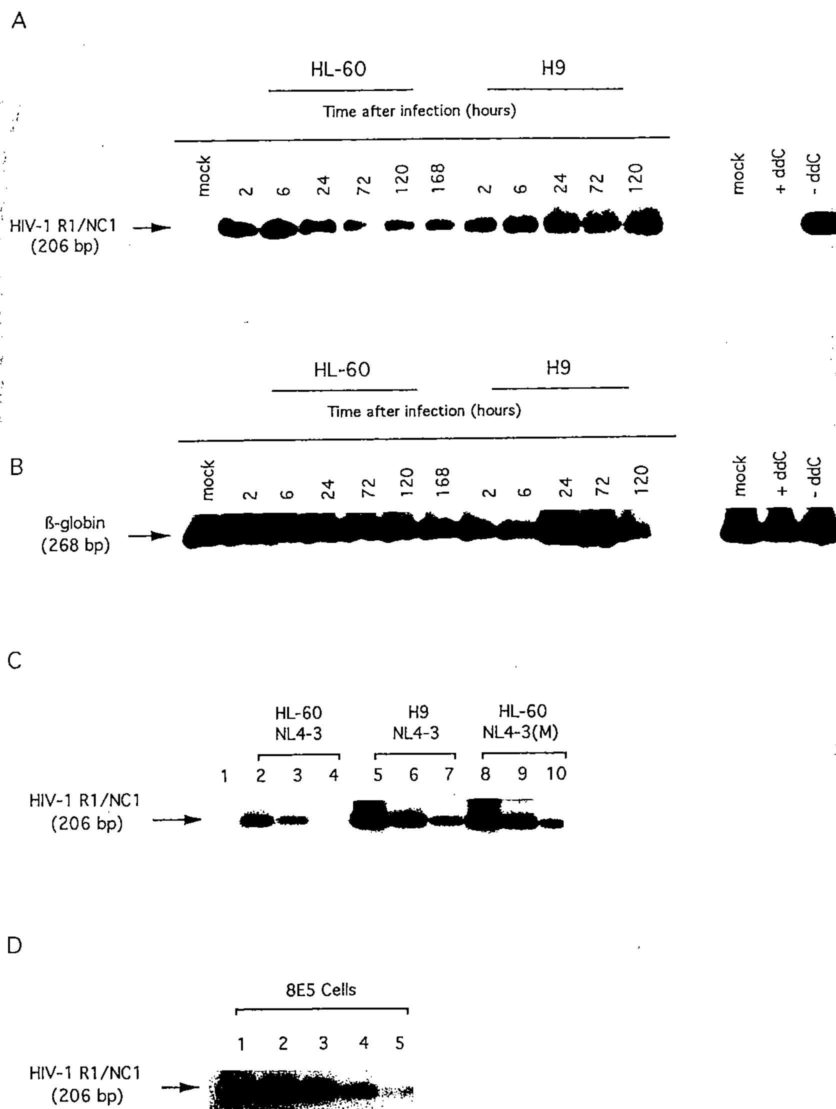

Figure III-8A-8D: Detection of newly reverse transcribed DNA in infected H9 and HL-60 cells. (continued on next page)

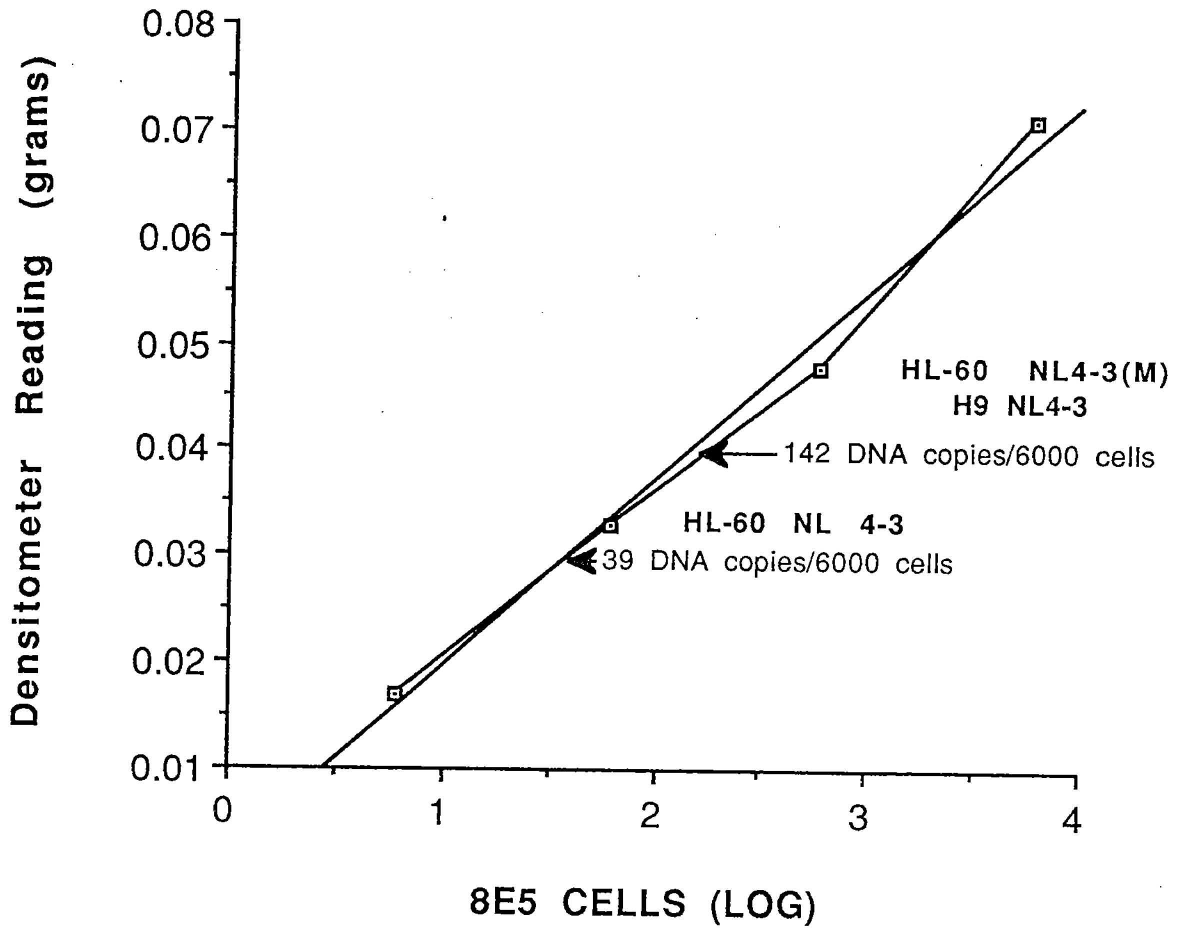

Figure III-8E: Detection of newly reverse transcribed DNA in infected H9 and HL-60 cells. (continued on next page)

Figure III-8: Detection of newly reverse transcribed DNA in infected H9 and HL-60 cells. H9 or HL-60 cells were infected with DNase treated NL4-3 at an MOI of 0.25 TCID/cell. Cell lysates were prepared at various time points after infection. In addition, HL-60 cells were pre-treated with 2μM ddC (+ ddC) for 15 minutes prior to addition of DNase treated NL4-3 and cell lysates prepared 2 hours after infection. The - ddC lanes represent cell lysates prepared 20 days after infection of HL-60 cells with NL4-3. (A) PCR analysis using primer pair R1/NC1 was performed on cell lysates (6×10^4 cells) to determine if viral DNA was present in HIV-1 infected cells. (B) To control for the amount of cellular DNA examined, lysates from H9 and HL-60 cells were amplified using beta-globin primer pair B1/B2. (C) Cell lysates from NL4-3 infected H9 and HL-60 cells and NL4-3(M) infected HL-60 cells (6 hours after infection) were serially diluted with uninfected HL-60 cell lysate. Lanes 2, 5, and 8 represent PCR products from undiluted cell lysates; lanes 3, 6, and 9 represent PCR products from a 1:10 dilution of cell lysate; and lanes 4, 7, and 10 represent PCR products from a 1:100 dilution of cell lysate. Lane 1 represents PCR products from undiluted cell lysate of mock infected cells. (D) Dilutions of 8E5 cells with uninfected HL-60 cells were used as a DNA standard (lanes 1 through 5 contain 60,000 8E5 cells, 6,000 8E5 cells, 600 8E5 cells, 60 8E5 cells, and 6 8E5 cells, respectively). (E) The densitometric values from (D) were plotted against the log of the number of 8E5 cells and the amount of viral DNA copies per infected H9 and HL-60 cells 6 hours after infection were extrapolated from this standard curve. Shown are the results from one of four identically performed experiments.

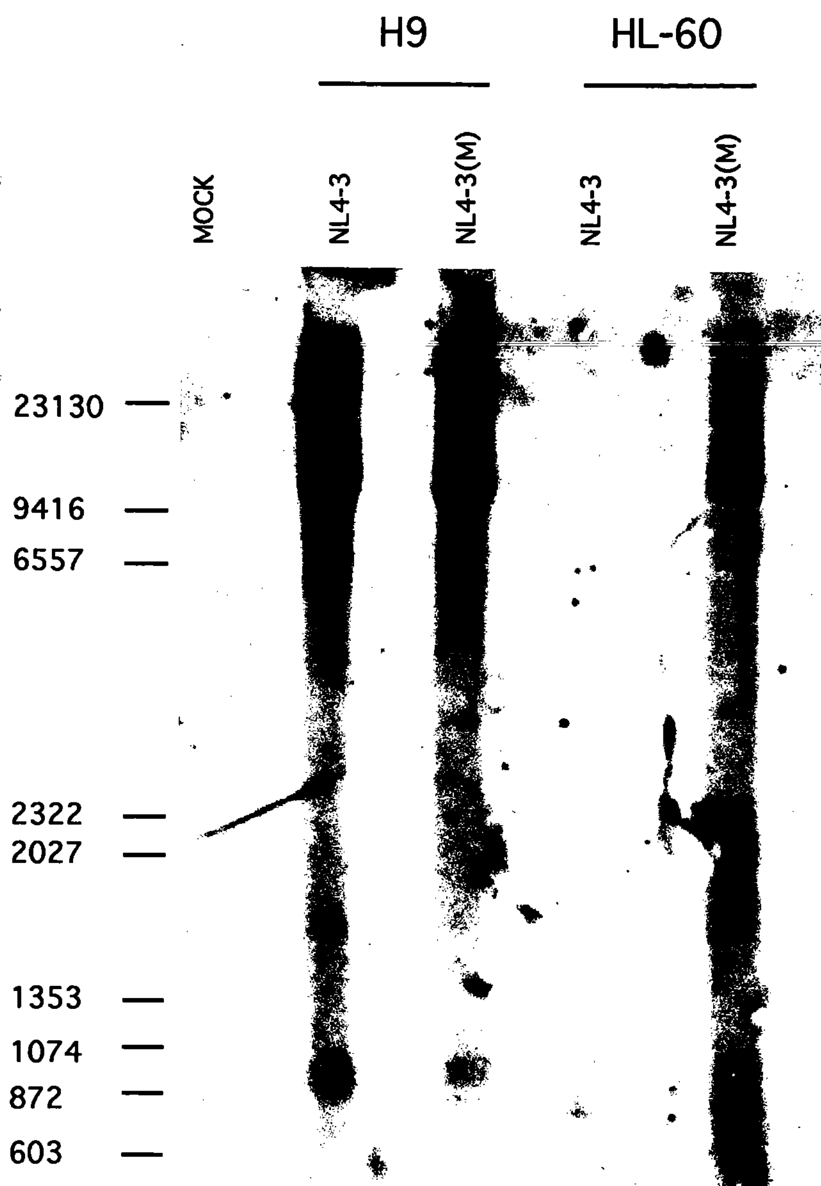

Figure III-9: Southern blot analysis of integrated proviruses. Genomic DNA's (20μg) from mock, NL4-3, or NL4-3(M) infected cultures harvested 5 days after infection were completely digested with Xho I, electrophoresed through a 0.7% agarose gel, and probed with a ^{32}P-labeled, HIV-1 specific DNA probe.

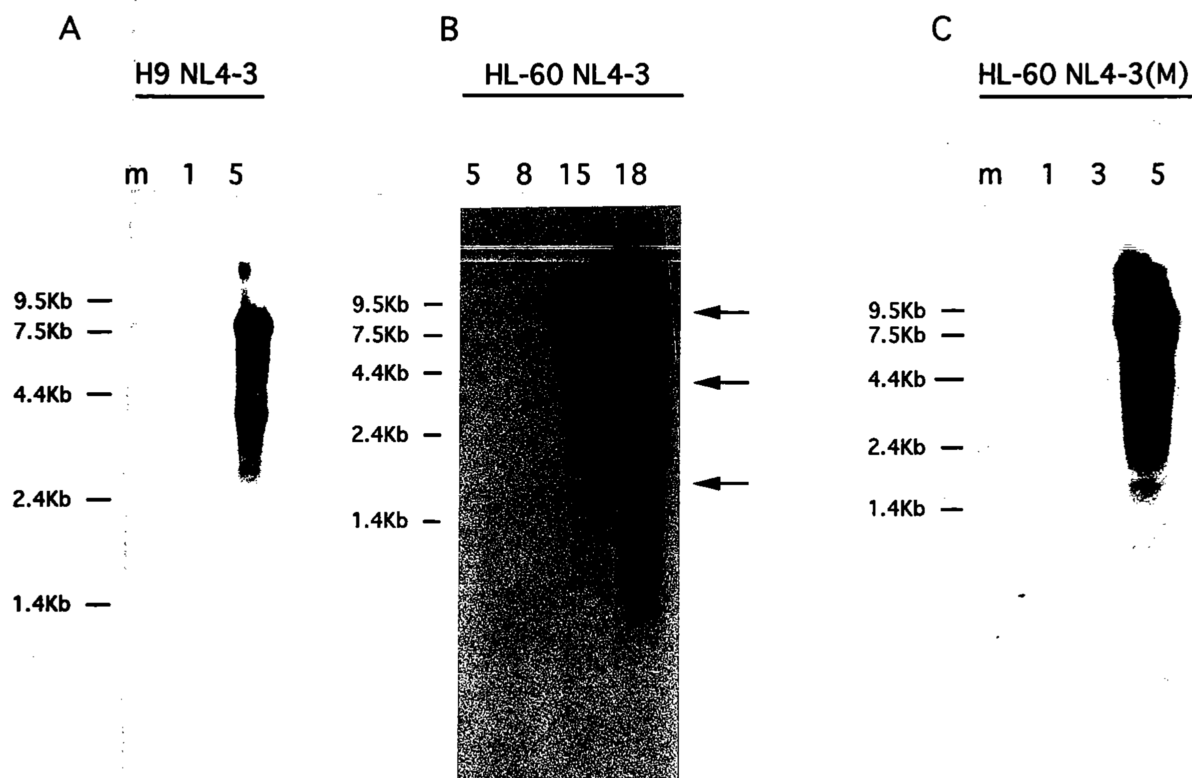

Figure III-10: Northern blot analysis of viral RNA. Total cellular RNA was prepared from NL4-3 infected H9 (A) and HL-60 (B) cultures and NL4-3(M) infected HL-60 (C) cultures at various days after infection as described in Materials and Methods. The numbers above the lanes designate the number of days after infection the RNA was prepared and the "m" designates RNA from mock infected cells. The arrows indicate the positions of the full length, singly, and multiply spliced RNA.

CHAPTER IV

ADAPTATION OF AN HIV-1 ISOLATE TO A HUMAN MYELOID CELL LINE EXTENDS THE TROPISM TO PRIMARY MACROPHAGES

INTRODUCTION

HIV-1 isolates exhibit distinct biological features including replication rate, cytopathic effect, serum neutralization, and cell tropism (21, 112, 169-175). Viruses isolated from asymptomatic patients replicate slowly in peripheral blood mononuclear cells (PBMC's) and very inefficiently, if at all, in transformed cell lines. Viruses with these characteristics have been called slow/low viruses. Viruses isolated from patients with severe immunodeficiency replicate rapidly in PBMC's and cell lines and produce high levels of reverse transcriptase. These viruses have been called rapid/high viruses (171). In the early stages of the disease primarily non-syncytia inducing, macrophage-tropic, slow/low viruses can be isolated from infected individuals. As the severity of the disease progresses, syncytia-inducing, T lymphocyte-tropic, rapid/high viruses are primarily isolated (171, 177). In addition, evidence strongly suggests that the changes in the biological features of the virus with time are signs of increased virulence in the host (177, 178, 180, 265). Thus, elucidation of the viral genes which control phenotypes associated with slow/low and rapid/high viruses may be important in understanding HIV-1 pathogenesis.

NL4-3 is a molecularly cloned HIV-1 recombinant isolate (104) that contains the 5' region of the HIV-1 isolate NY5 (nt 1 to nt 5740) and the 3' region of the HIV-1 isolate LAV (nt 5740 to nt 9709). NL4-3 productively infects both primary T lymphocytes and transformed T cell lines (104). I recently characterized NL4-3(M), a variant virus, isolated from HL-60 cells during the lytic phase of infection with NL4-3 (Chapter III). NL4-3(M), like NL4-3, has the capacity to rapidly kill H9 cells and has acquired the capacity to rapidly kill the human bipotential, myeloid cell lines, HL-60 and PLB985.

Cells of the myeloid lineage give rise to macrophages and thus viral isolates which can efficiently replicate in HL-60 cells may also be capable of replicating in macrophages. To further characterize the tropism of NL4-3(M), the ability of NL4-3(M), NL4-3, and Ba-L, a macrophage tropic HIV-1 isolate (202), to infect monocyte-derived macrophages (MDM) and peripheral blood lymphocytes (PBL) cultures was examined.

MATERIALS AND METHODS

Cells, viruses and infections. Human peripheral blood mononuclear cells were obtained by leukopheresis from normal, seronegative volunteers, layered onto lymphocyte separation medium (LSM, Organon Teknika), and centrifuged for 30 minutes at 1500g. Cells were separated into lymphocyte and monocyte fractions by counterflow centrifugal elutriation (267). Characterization by flow cytometry demonstrated the lymphocyte fractions to be 95-97% positive for CD2 or CD3; the monocyte fractions were >89% positive for CD14. Lymphocytes (PBL) were stimulated with 5 μg/ml Con A for 48 hours, and maintained in RPMI 1640 containing 10% fetal bovine serum, and 20 units/ml of interleukin 2 (Genzyme, Cambridge, MA). Monocytes were differentiated to macrophages by culture in DMEM containing 10% human AB serum and penicillin (100 units/ml)/streptomycin (100 μg/ml) on plastic plates for 21 days with non adherent cells removed every third day.

MDM and PBL cultures were infected with DNase treated stocks (Chapter III) of NL4-3, NL4-3(M), or NL4-3envA at a multiplicity of infection (MOI) of 0.2 to 0.5 C8166 tissue culture infectious dose (TCID)/cell. HIV Ba-L (202), an HIV-1 isolate from a primary culture of plastic-adherent, non-specific esterase positive cells of human infant lung tissue (ABI, Columbia, Maryland), was inoculated at $10^{5.67}$ $TCID_{50}$ units. All cultures were maintained by changing the medium every 72 hours for a total of 21 days and cell-free supernatants were collected. Cell-free supernatants collected at 14 and 21 days after infection were assayed for p24 using an antigen capture Elisa method (Coulter HIV-1 p24 Antigen Assay, Hilaleah, FL).

Co-culture assay. Cultures of NL4-3, NL4-3(M), or Ba-L, infected PBL and MDM, or 1.0ml of cell-free supernatants from these cultures were co-cultured with 2.5 x 10^5 C8166 cells 21 days after infection to examine expression of viral envelope proteins

on the surface of infected cells and to examine release of virus into the medium of infected cultures. C8166 cells are an HTLV-I infected indicator cell line (244) which readily forms syncytia with HIV-1 infected cells. The cultures were scored for syncytia formation 48 hours after co-cultivation.

Polymerase chain reaction (PCR) analysis. Cell lysates were prepared from MDM and PBL cultures 21 days after infection by washing the cells twice with cold phosphate buffered saline (PBS) and suspending the cells at a density of 6×10^6 cells/ml in PCR lysis buffer (50mM KCl, 10mM Tris-HCl (pH8.3), 2.5mM $MgCl_2$, 0.1mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20, and 6ng/ml Proteinase K). Samples were incubated at 56°C for one to two hours, heated at 100°C for ten minutes, and stored at -20°C. The HIV-1 specific primer pair used was: pol1, 5'-GATACAGGAGCAGATGATACAG-3' (nt 2325 to nt 2347) and pol2, 5'-CTGGAGTATTGTATGGATTTTCA-3' (nt 2705 to nt 2682). This primer pair is predicted to amplify a 380 base pair fragment of the viral polymerase gene. Beta-globin primers B1 and B2 (Chapter III) which are predicted to amplify a 268 base pair fragment of the single copy beta-globin gene were used as internal amplification standards. PCR reactions were performed using 1.0μM of each primer in a 50μl reaction volume containing each of the four deoxynucleoside triphosphates at 200μM, 10mM Tris-HCl (pH8.3), 50mM KCl. 1.5mM $MgCl_2$, 0.001% gelatin, 1.25 units of *AmpliTaq* DNA Polymerase (Perkin Elmer Cetus), and cell lysate of 6×10^4 cells. The reactions were overlaid with a drop of mineral oil and subjected to 35 cycles (denatured for 45 seconds at 95°C, annealing for 2 minutes at 57°C, extension for 3 minutes at 72°C, and a final 10 minute extension at the 35th cycle) in a Thermolyne thermocycler.

Southern hybridization analysis. Amplified products were electrophoresed through 3.0% agarose gels (see Chapter III, Materials and Methods). The gels were treated for 10 minutes with 0.25N HCl and the DNA was transferred to nylon membranes (Zetabind, CUNO) in 0.4N NaOH for 2 to 18 hours. Following 3 hours of pre-hybridization at 65°C in 4X SSCP, 1X Denhardt's, 0.5% SDS, and 250µg/ml sheared salmon sperm DNA, the membranes were hybridized overnight at 65°C in buffer containing 4X SSCP, 1X Denhardt's, 1.0% SDS, 10% sodium dextran sulphate, and 10^7 cpm of ^{32}P-labeled (Random Primers labeling kit, Bethesda Research Laboratories) DNA from a 4314bp Hind III fragment of pNL4-3 (nt 1712 to nt 6026). Following hybridization, membranes were washed in 3X SSCP, 4X Denhardt's, 0.1% SDS briefly at room temperature, in 3X SSCP/0.1% SDS for fifteen minutes at 65°C, in 1X SSCP/0.1% SDS for fifteen minutes at 65°C, and in 0.1X SSCP/0.1% SDS for 15 minutes at 65°C and then subjected to autoradiography (see Chapter III).

Recombinant virus construction. Episomal DNA was collected from NL4-3(M) infected HL-60 cells (or mock infected cells) 48 hours after infection using the method outlined by Hirt (268). Briefly, 2×10^7 to 1×10^8 cells were pelleted, washed with PBS and suspended in 1 ml of buffer (10mM Tris-HCl (pH7.5), 10mM EDTA, 5mM EGTA). One ml of buffer containing 2% SDS was added, the samples incubated for one hour at 37°C and after adding 0.5ml of 5M NaCl, incubated overnight at 4°C. The cell lysates were centrifuged at 17,000g, 4°C for 30 minutes. The supernatants were extracted with phenol and then chloroform:isoamyl alcohol (24:1). Episomal DNA was precipitated with two volumes of 100% ethanol on ice for 30 minutes. The DNA samples were pelleted by centrifugation at 25,000g, for 30 minutes at 4°C, washed with 70% ethanol and suspended in 200µl of 10mM Tris-HCl (pH7.5), 5mM EDTA.

Approximately 1µg of episomal DNA was used for PCR amplification. The pNL4-3 specific primer pair, env 1, 5'-TATGGGGTACCTGTGTGGAAGG-3' (nt 6338 to nt 6359) and env 2, 5'-TTCTAGGTCTCGAGATACTGCTC-3' (nt 8878 to nt 8900) was

used to amplify a 2544 bp product containing the majority of the envelope (*env*) gene of the virus. The product was gel purified and cloned using the TA cloning system (Invitrogen). Four of the 120 envelope containing clones were chosen for further analysis. The Kpn I-Xho I fragments were excised from the plasmids and placed back into the corresponding position (nt 6343 to nt 8887) in pNL4-3. The recombinant viral constructs were transfected into H9 cells by the method of DEAE-dextran (Chapter III). Virus harvested from transfected H9 cells was used to infect H9 and HL-60 cells. The viabilities of the cultures were monitored by vital dye exclusion.

Electron Microscopy. HIV-infected or uninfected MDM cultures were harvested 21 days after infection. HIV-infected and uninfected HL-60 and H9 cell cultures were harvested 7 days after infection. All cell cultures were washed with PBS and immediately fixed in 2.5% glutaraldehyde in PBS overnight at 4°C. Attached cells, suspended by gentle scraping, and unattached H9 and HL-60 cells were processed into Spurrs' epoxy after solidifying into agar. Thin sections were stained with uranyl actetate and lead citrate and examined with a Zeiss EM 10A^{R} at 60 kV.

Sequencing of regions of the envelope gene of NL4-3envA. Sequencing of pNL4-3envA was performed using reagents provided by the U. S. Biochemical Sequenase Kit and sequenced according to the manufacturers instructions. Briefly, plasmid DNA (5μ g) was alkaline denatured (0.2N NaOH) for 5 minutes at room temperature and then ethanol precipitated. The dried pellet was suspended in water and the primer (0.5pmol/μl) annealed, the DNA labeled, and the reactions terminated. The samples were separated by electrophoresis on a 6% polyacrylamide gel.

RESULTS

Infection of MDM and PBL cultures. MDM and PBL cultures were infected with DNase treated NL4-3 and NL4-3(M), and untreated Ba-L to determine the host range of NL4-3(M). The viral isolate Ba-L was used as a control for productive infection of MDM cultures. No cytopathic effects were observed in any of the infected or control MDM cultures 21 days after infection. Conversely, all infected PBL cultures showed varying degrees of syncytia and cell death 21 days after infection with either NL4-3, NL4-3(M), or Ba-L. NL4-3(M) infection of PBL's induced larger, multinucleated syncytia compared to the syncytia induced by NL4-3 (data not shown).

To determine if viral DNA was present 21 days after infection of PBL and MDM cultures, polymerase chain reaction (PCR) amplifications of cell lysates from PBL and MDM cultures infected with either NL4-3, NL4-3(M), or Ba-L were assayed. When an HIV-1 specific primer pair (pol 1/pol 2) was used for amplification, a 380 bp region of the viral polymerase gene was detected in DNA from all infected PBL cultures (Fig. IV-1A). Viral DNA was detected in cell lysates from NL4-3(M) and Ba-L infected MDM but not in cell lysates from NL4-3 infected MDM (Fig. IV-1A). As a control for the amount of cellular DNA present in the amplification reactions, the single copy cellular gene for beta-globin (primer pair: B1/B2) was amplified (Fig. IV-1B). These results indicate that unlike NL4-3, NL4-3(M) and Ba-L were able to enter and be completely reverse transcribed in macrophages. In addition, the viral DNA persisted for 21 days after infection.

Virus expression and release. To determine if the viral envelope gene was being expressed on the surface of infected cells, PBL and MDM cultures infected with NL4-3, NL4-3(M), or Ba-L were co-cultured with C8166 cells. The cultures were scored for syncytia formation 48 hours after co-cultivation. Syncytia formation was observed in the NL4-3 and NL4-3(M) infected PBL co-cultures and in the NL4-3(M) infected MDM co-cultures but not in the NL4-3 infected MDM co-cultures (Table IV-1). To determine if

virus was being released from the NL4-3, NL4-3(M), or Ba-L infected PBL or MDM cultures, 1.0ml of cell free supernatant was used to infect C8166 cells and scored for syncytia formation. Supernatant from NL4-3 and NL4-3(M) infected PBL cultures and NL4-3(M) infected MDM cultures caused syncytia formation, while supernatant from NL4-3 infected MDM did not cause syncytia. As previously demonstrated, many macrophage tropic isolates do not productively infect T cell lines (13, 16, 170). It was not surprising that syncytia formation was not observed in Ba-L infected cultures. Therefore, NL4-3(M) productively infected both PBL and MDM while NL4-3 productively infected only PBL cultures.

Electron microscopic analysis of infected cultures. To examine the virion structure of NL4-3(M) in macrophages compared to HL-60 and H9 cells, electron microscopy was performed. The results are shown in Figure IV-2. The number of virions per cell appears to be lower in NL4-3(M) infected macrophages (Fig. IV-2D) and HL-60 cells (Fig. IV-2B) as compared to NL4-3(M) infected H9 cells (Fig. IV-2C) or Ba-L infected macrophages (Fig. IV-2A). Typical of MDM, in addition to viral production at the plasma membrane, virus was also being formed and collecting within cytoplasmic vacuoles (Fig IV-2A). The overall morphogenesis and morphology was typical for HIV in all infected cultures.

Recombinant virus containing the envelope of the NL4-3(M). To determine if the envelope gene of NL4-3(M) conferred the ability of NL4-3(M) to replicate in MDM cultures, I have generated a recombinant virus that contains the *env* gene of NL4-3(M). The envelope gene of NL4-3(M) was amplified from Hirt DNA preparations of NL4-3(M) infected HL-60 cells. The primers used for amplification of the *env* gene are shown in Figure IV-3A. A 2544 nucleotide fragment was amplified, isolated, cleaved with Kpn I and Xho I, and inserted into the corresponding restriction sites in a molecular clone of NL4-3. Four independent recombinants produced infectious virus when transfected into H9 cells (NL4-3envA, NL4-3envB, NL4-3envC, and NL4-3envD). These viruses were

used to infect both H9 and HL-60 cells. The viabilities of H9 and HL-60 cultures after infection with recombinant NL4-3envA were determined and are shown in Figure IV-3B. The results with the remaining three recombinants were similar (data not shown). All of the recombinant viruses produced cytopathic effects in HL-60 cells that were similar to that of NL4-3 and unlike that of the highly cytopathic virus NL4-3(M). The mean survival time (MS_{50}) of NL4-3 infected HL-60 cells was 15 days which varied from the 22 days observed previously (Chapters II and III). Nevertheless, the MS_{50} of NL4-3 and NL4-3envA infected HL-60 cells was significantly delayed as compared to the MS_{50} (5 days) of NL4-3(M) infected HL-60 cells (Fig. IV-3B). These data indicate that the envelope gene of NL4-3(M) is not the sole determinant of the rapid cytopathic effect of NL4-3(M) in HL-60 cells.

Infection of MDM cultures with NL4-3envA. To determine if the envelope recombinant, NL4-3envA, could replicate in MDM cultures, cell-free supernatants were collected from transfected H9 cells and used to infect MDM cells. In addition, cell-free supernatants of NL4-3, NL4-3(M), and Ba-L were used to infect new MDM cultures. Cell lysates were made from these cultures 21 days after infection and PCR amplification was performed using primers specific for the HIV-1 polymerase gene (Fig. IV-4A) and control primers specific for beta-globin (Fig. IV-4B). As shown in Figure IV-2, HIV-1 DNA amplification products could be detected in NL4-3(M), and Ba-L infected cultures but not in NL4-3 or mock infected cultures (Fig. IV-4A). Surprisingly, HIV-1 DNA amplification products were also detected in NL4-3envA infected MDM cells. These data demonstrate that although the *env* gene of NL4-3(M) is not the sole determinant of the cytopathic effect of the virus in HL-60 cells, it is capable of conferring viral replication in MDM cells. DNA sequencing of the V3 region of the envelope gene of NL4-3envA revealed that the V3 loop was identical to that of NL4-3 (Fig. IV-5A) but that there were differences in sequences outside the V3 loop which resulted in an in-frame deletion of the NL4-3 sequence that effectively converted two glycine residues (nt 7310-7315) to a

glutamine residue (Fig. IV-5B). These data suggest regions outside the V3 loop of the *env* gene are responsible for the macrophage tropism of NL4-3(M).

To determine if extracellular virus was being produced, culture supernatants were collected 14 and 21 days after infection and cell-free p24 values determined using an antigen capture ELISA assay. The p24 values (Table IV-2) indicate that virus is released from NL4-3(M), NL4-3envA, and Ba-L infected macrophages. However, the amount of extracellular p24 released from NL4-3envA infected MDM cells was much lower and decreased with time (41pg/ml on day 21) compared to the p24 values observed for NL4-3(M) and Ba-L infected MDM cells (8886pg/ml and 10246pg/ml on day 21, respectively). These results indicate that although the envelope gene of NL4-3(M) allowed viral entry into macrophages, another viral determinant was involved in efficient viral replication. This is further supported by the data demonstrating that unlike NL4-3(M), NL4-3envA did not cause rapid cell death in HL-60 cells (Fig. IV-3B).

DISCUSSION

I have determined that the viral variant, NL4-3(M), unlike NL4-3, is capable of productively infecting macrophages. Furthermore, this change in cell tropism can be attributed to the envelope gene of NL4-3(M), since the envelope recombinant virus, NL4-3envA, is capable of replicating in macrophages. Although several investigators have demonstrated that macrophage tropism is determined primarily by portions of the V3 loop of the envelope gene (13, 16, 17, 20, 174, 269), the sequence of the V3 loop of NL4-3envA and NL4-3 were identical (Fig. IV-5A). This suggests that the ability of NL4-3(M) and NL4-3envA to infect macrophages is due to changes in the envelope gene of the parental NL4-3 which lie outside the V3 loop. An alteration spanning nucleotides 7310-7315 in the envelope gene resulted in the replacement of 2 glycine residues by a glutamine residue in NL4-3envA. This change, downstream of the V3 loop, could alter the conformation of the gp120 protein and thus affect viral infectivity. These results are consistent with recent reports that sequences throughout the *env* gene are important in determining the biological properties of the virus and can influence both the conformation of gp120 and viral infectivity (20, 270-272).

The ability of NL4-3envA to replicate in MDM cultures is unexpected since its biological phenotype in HL-60 cells is similar to that of NL4-3. This suggests that envelope determinants that control viral entry into MDM are different from the determinants that control the cytopathic phenotype in HL-60 cells. In addition, the production of progeny virus from NL4-3envA infected MDM cultures is much lower than from NL4-3(M) or Ba-L infected MDM cultures. This further indicates that there are additional changes in the NL4-3(M) genome which influence virus production in macrophages. These data are consistent with recent reports that macrophage tropism may be restricted in more than one stage of virus replication and is thus controlled by other factors (264, 273) in addition to the envelope gene. In addition to the envelope gene

NL4-3envA also contains exon 2 of the *rev* gene. Since this exon encodes the Rev functional domain (312), differences between the Rev protein of NL4-3(M) and NL4-3 are not likely to be involved in the differences in cytopathicity of the viruses in HL-60 cells and in the differences in viral production in macrophages.

Viral cell tropism is thus determined by two processes: virus entry and efficiency of progeny production. The virus must bind to its receptor on the cell surface and penetrate the target cell. After entry, intracellular events then determine the levels of virus production. As shown in these studies, each of these processes can affect the kinetics of replication in an infected cell. The viral variant described here is unique in that it is capable of replicating in primary macrophages and myeloid cell lines. Since the myeloid cell lineage gives rise to both macrophages and granulocytes, infection of this cell type could have a profound affect on disease progression. Furthermore, the identification of cellular and viral determinants which control myeloid and macrophage cell tropism may provide important insight into HIV-1 pathogenesis.

FIGURES AND TABLES

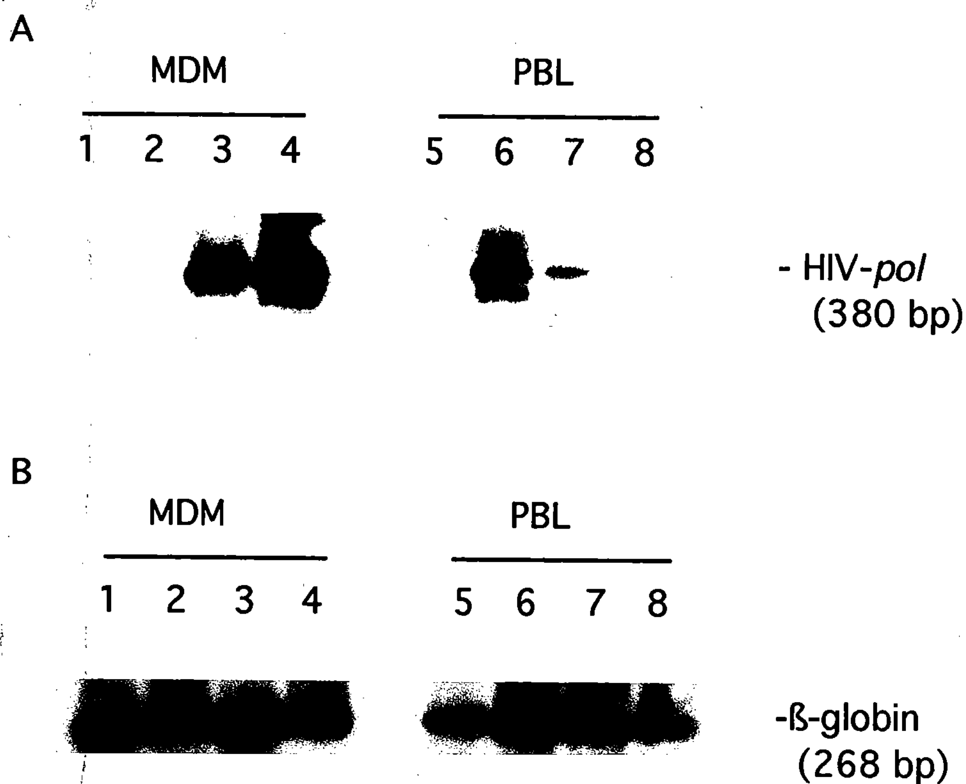

Figure IV-1: **Detection of viral DNA.** MDM and PBL cultures were infected with either NL4-3 (lanes 2 and 6), NL4-3(M) (lanes 3 and 7), Ba-L (lanes 4 and 8), or mock infected (lanes 1 and 5) and cell lysates were prepared 21 days after infection. (A) To determine the presence of viral DNA, PCR analysis was done using the HIV-1 specific primer pair pol 1: 5'AGATACAGGAGCAGATGATACAG 3' (nt 2325 to nt 2358) and pol 2: 5'ATTTTTCCTTCCTTTTCCATTTC 3' (nt 2667 to nt 2690). (B) The same cell lysates were amplified using a primer pair specific for beta-globin as a control.

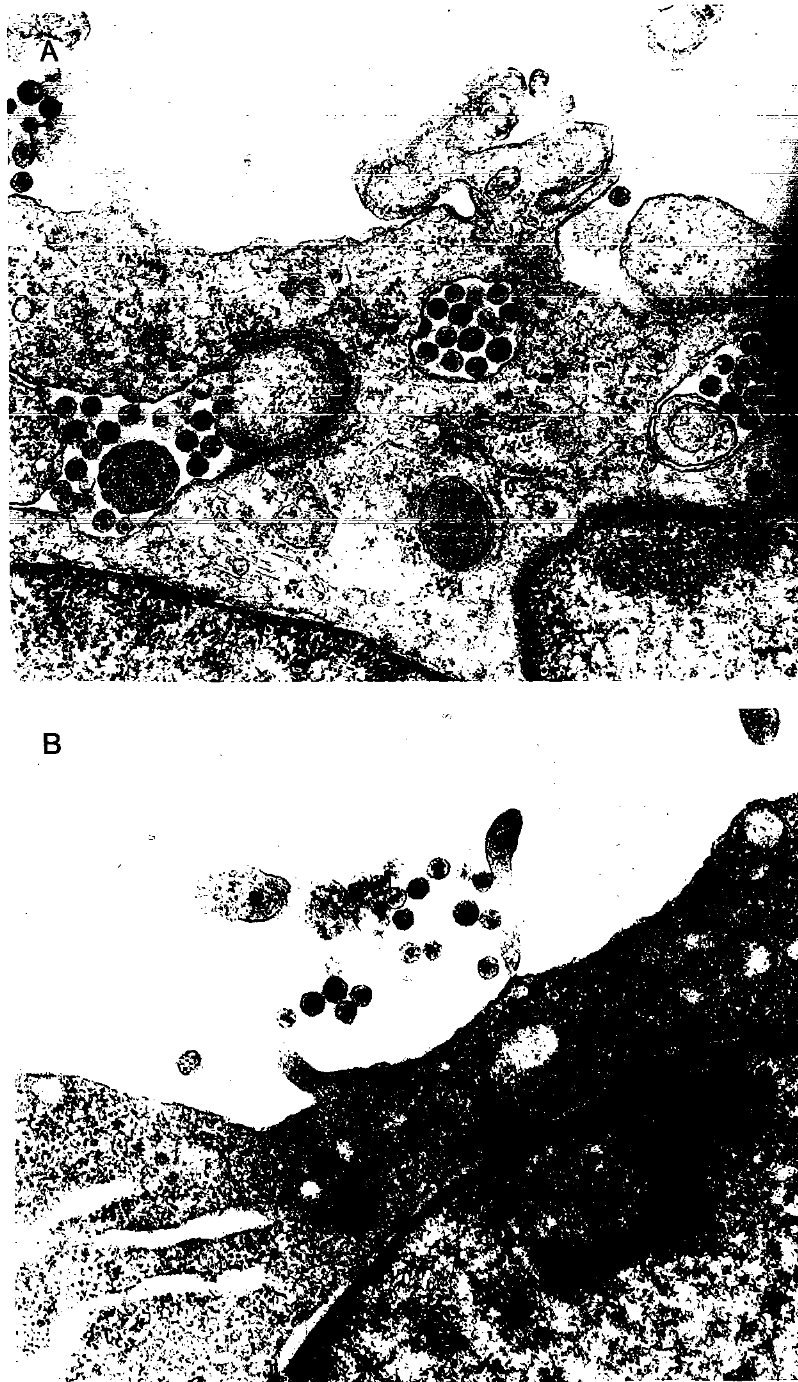

Figure IV-2: **Electron microscopy.** (A) Electron microscopy was performed on a Ba-L infected MDM culture. Typical mature and budding HIV-1 particles are located within deep invaginations of the cell surface (50,000X). (B) In an NL4-3(M) infected HL-60 culture a rare cluster of mature particles is present near the cell surface (53,000X). (continued on next page)

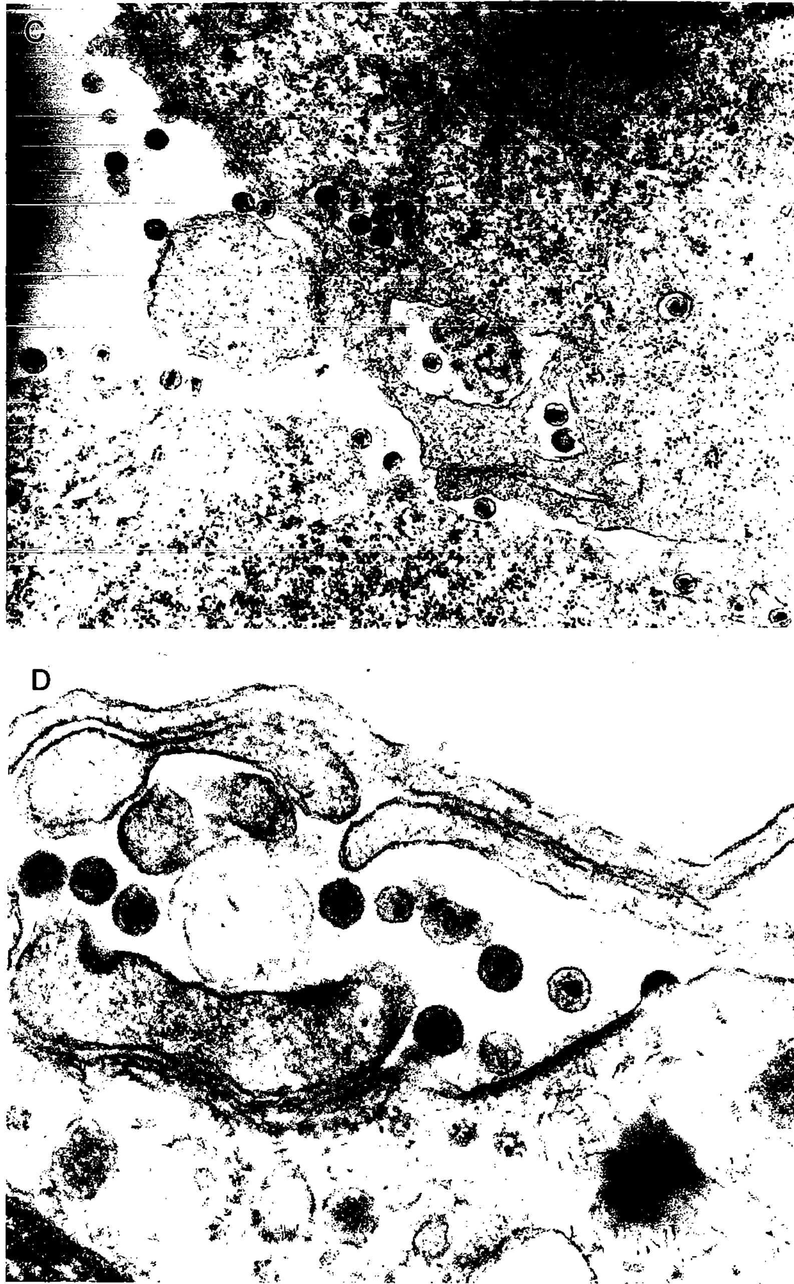

Figure IV-2: **Electron microscopy.** (continued from previous page) (C) Electron microscopy was performed on an NL4-3(M) infected H9 culture. Shown are two cells with many virions either on the surface or within cytoplasmic vacuoles located near the cell surface (40,000X). (D) NL4-3(M) infection of an MDM culture shows several mature particles and a budding particle covered by cell surface folds present in the infected cell. A subplasmalemmal density typical of mononuclear phagocytes is adjacent to the budding particle (60,000X).

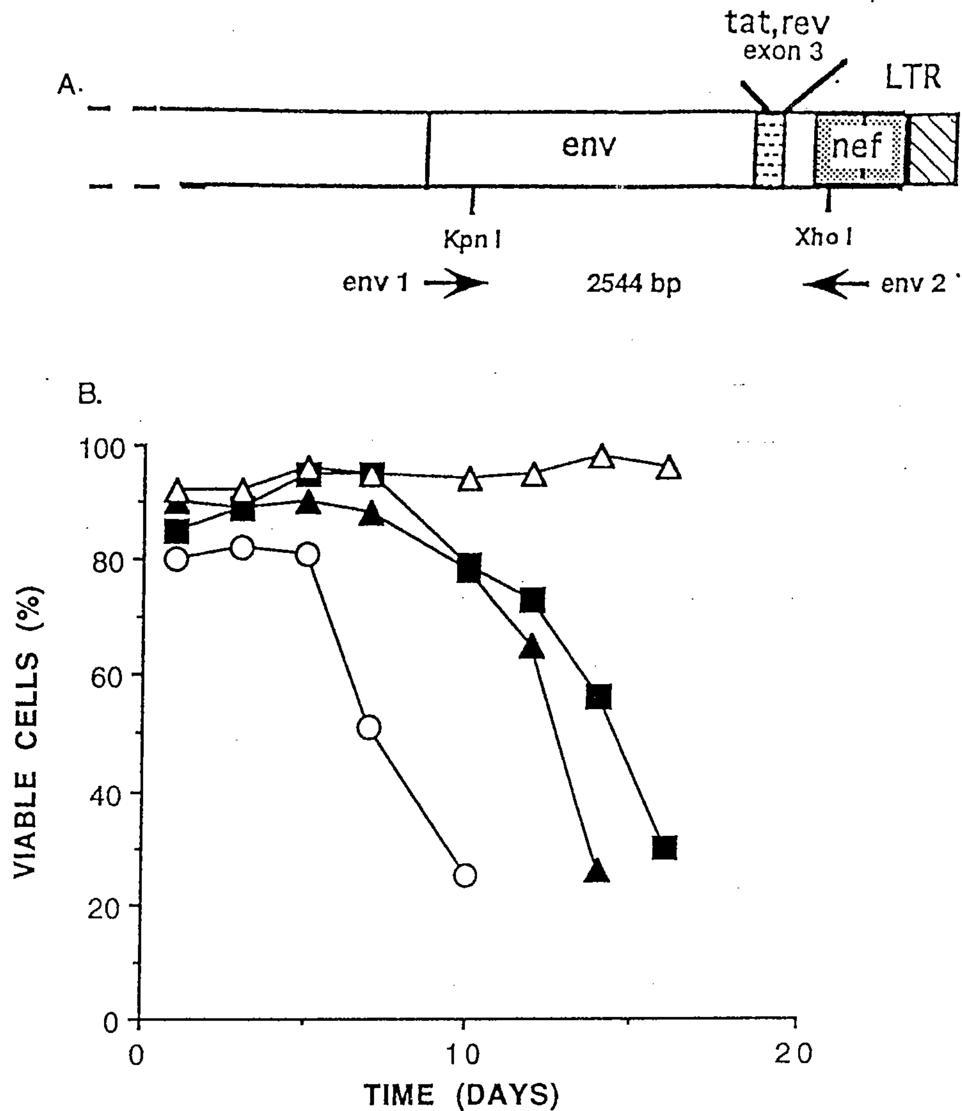

Figure IV-3: **Envelope gene.** (A) The primer pair represented in the diagram was used to amplify a 2544 base pair fragment of the *env* gene derived from NL4-3(M) infected HL-60 cells. The Kpn I to Xho I *env* fragment was isolated and used to replace the env fragment of the parental NL4-3. Recombinant virus, NL4-3envA, was harvested from DEAE-dextran transfected H9 cells. (B) HL-60 cells were infected with NL4-3envA (-Δ-), NL4-3 (-■-), or NL4-3(M) (-O-) at a MOI=0.2 TCID/cell in the presence of 2μg/ml polybrene. The open triangle represents mock infected HL-60 cells. The viabilities of the cultures were monitored by trypan blue dye exclusion.

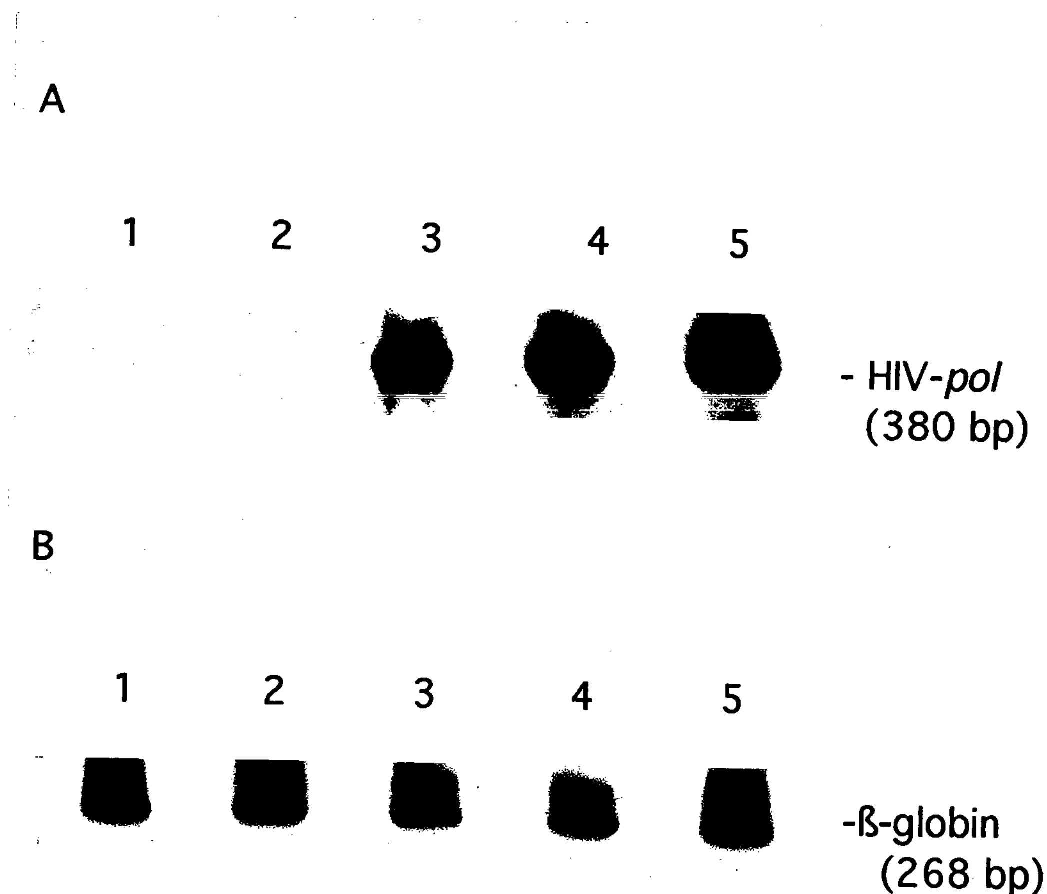

Figure IV-4: **Infection of MDM cultures.** Cell-free supernatants were harvested from either NL4-3envA transfected or mock infected H9 cells or NL4-3, NL4-3(M), or Ba-L to determine if the envelope gene of NL4-3(M) controlled macrophage tropism. MDM cultures were then infected with DNase treated supernatants at a MOI of 0.25 TCID/cell in the presence of 2μg/ml polybrene. Cell lysates were prepared 21 days after infection from NL4-3 (lane 2), NL4-3(M) (lane 3), NL4-3envA (lane 4), Ba-L (lane 5) or mock (lane 1) infected MDMs. PCR analysis was performed on the lysates using the HIV specific primer pair pol1/pol2 (A) and the beta-globin primer pair B1/B2 (B).

A.

7085

TCT GTAGAAATTAAT[TGTACAAGACCCAACAACAATACAAGAAAAAGTAT
TCTGTAGAAATTAAT[TGTACAAGACCCAACAACAATACAAGAAAAAGTAT

7136

CCGTATCCAGAGGGGACCAGGGAGAGCATTTGTTACAATAGGAAAAATA
CCGTATCCAGAGGGGACCAGGGAGAGCATTTGTTACAATAGGAAAAATA

7187

GGAAATATGAGACAAGCACATTGT]AACATTAGTAGAGCAAAATGGAAT
GGAAATATGAGACAAGCACATTGT]AACATTAGTAGAGCAAAATGGAAT

B.

7161

CATTTGTTACAATAGGAAAAATAGGAAATATGAGACAAGCACATTGTAAC
CATTTGTTACAATAGGAAAAATAGGAAATATGAGACAAGCACATTGTAAC

7212

ATTAGTAGAGCAAAATGGAATGCCACTTTAAAACAGATAGCTAGCAAATTA
ATTAGTAGAGCAAAATGGAATGCCACTTTAAAACAGATAGCTAGCAAATTA

7264

AGAGAACAATTTGGAAATAATAAAACAATCTTTAAGCAATCCTCAGGAGGG
AGAGAACAATTTGGAAATAATAAAACAATCTTTAAGCAATCCTCA--**GAG**----

7316

GACCCCGAAATTCTAACGCACAGTTTTAATTGTGGAGGGGAATTTTTCTAC
GACCCCGAAATTCTAACGCACAGTTTTAATTGTGGAGGGGAATTTTTCTAC

7368

TGTAATT
TGTAATT

Figure IV-5: **Nucleotide sequence of portions of the envelope gene of NL4-3envA.** (A) The V3 loop sequence of NL4-3 (top line) is compared to that of NL4-3envA (bottom line). The V3 loop is designated using brackets. (B) The C5 (constant region 5) through the C8 (constant region 8) sequences of NL4-3 (top line) are compared to NL4-3envA (bottom line). Numbers to the left indicate nucleotide positions in NL4-3. Sequence differences are indicated in bold. Deletion of three guanine nucleotides are designated by dashed lines.

Table IV-1. Infection of MDM and PBL Cultures.

Cell Type[a]	PCR ANALYSIS (21 days p.i.)[b]	Syncytia Formation with C8166 Cells[c]	
		co-culture	cell-free supernatant
MDMs			
Uninfected	-	-	-
NL4-3	-	-	-
NL4-3(M)	+	+	+
Ba-L	+	-	-
PBLs			
Uninfected	-	-	-
NL4-3	+	+	+
NL4-3(M)	+	+	+
Ba-L	+	-	-

[a] MDM and PBL cultures (10^6 cells/ml) were infected with NL4-3, NL4-3(M), Ba-L, or mock infected on day 0 and cultured for 21 days. MDM cells were maintained in DMEM supplemented with 10% human AB sera and PBL cells were maintained in RPMI supplemented with 10% fetal bovine sera and 20 units/ml IL-2. Culture medium was changed every third day.

[b] Cell lysates were prepared from duplicate cultures and assayed by PCR amplification for the presence of viral DNA (+ = viral DNA detected; - = no viral DNA detected).

[c] C8166 cells were co-cultures with cells or cell-free supernatants from MDM and PBL cultures (+ represents >5 multinucleated cells/well; - represents <2 multinucleated cells/well).

Table IV-2. Detection of Extracellular p24 Core Antigen from Infected Macrophages.

Infected MDMs[a]	p24 antigen (pg/ml)[b]	
	DAY 14	DAY 21
Mock	n[c]	n
NL4-3	n	n
NL4-3envA	197	41
NL4-3(M)	2691	8886
Ba-L	2137	10246

[a] MDMs (2 X 10^6) were infected with DNase treated viral stocks on day 0 . Twenty-four hours after infection cultures were washed twice with PBS and maintained in DMEM supplemented with 10% human AB sera. Culture medium was changed every third day.

[b] Cultures were washed with PBS the day before sample collection. Cell-free supernatants were taken 14 and 21 days after infection. Enzyme-linked immunosorbant assay for extracellular p24 core antigen, using the Coulter HIV-1 antigen kit, was performed.

[c] n = none detected, below or equal to background values (<7pg/ml) detected in medium.

CHAPTER V

SUMMARY

Introduction.

A variety of hematologic abnormalities have been described during the course of HIV-1 infection including neutropenia, thrombocytopenia, and anemia (236, 237). These abnormalities increase in severity as the disease progresses (236, 237). Bearing in mind that the hematopoietic stem cells are common progenitors to the myeloid and lymphoid lineages, a cytotoxic effect of HIV-1 on the ancestral hempatopoietic progenitors could account not only for these hematological abnormalities, but also for the incapacity to compensate the constant and progressive decrease in $CD4^{+}$ T lymphocytes. In addition, HIV-1 induced impairment of differentiation or cellular function could also contribute to disease progression. This final Chapter will review the major findings of this dissertation in light of the current literature.

Effects of HIV on Myeloid Differentiation.

It is suggested that HIV-1 infection has a direct role in causing the hematological abnormalities observed in infected individuals since a reduced number of hematopoietic progenitor cells are found in AIDS/ARC patients (212, 236). In addition, infection of human $CD34^{+}$ cells isolated from infected individuals causes a loss in the ability of these cells to form colonies of differentiated progeny. Infected bone marrow stromal cells (211), immune mediated suppression (221), and production of inhibitory molecules and cytokines from infected accessory cells (211, 222-224), may all play a role in the abnormal hematopoiesis observed in AIDS patients. I have demonstrated that *in vitro* HIV-1 infection of myeloid cells does not affect the morphological or functional differentiation of these cells (Chapter II). These results are supported by data from several investigators examining the effects of HIV-1 infection on monocytic differentiation (215, 230, 262). Although a normal pattern of differentiation was observed for HIV-infected myeloid cells *in vitro*, this does not rule out the possibility that the differentiation of these cells may be altered *in vivo*. *In vivo*, cellular differentiation is influenced by the microenvironment,

accessory cells, as well as stimulatory signals and cytokines. It is possible that the stage of cellular differentiation at which infection occurs may also determine the outcome of cellular differentiation and function. The system I have studied is lineage committed and HIV-1 infection may not affect further differentiation of this cell type. Differentiation and function of multipotent cells or the self-renewing stem cell, however, may be inhibited by HIV-1 infection.

Effects on the Immunological Function of HIV-1 Infected Cells.

Recently, it has been shown that HIV-infected monocytes isolated from infected individuals have normal (1) microbicidal activity against several unrelated pathogens (*Candida albicans, Aspergillus fumigatus, Toxoplasma gondii, Chlamydia psittaci*) (274-276), (2) phagocytositic activity of latex beads or infectious microbes (274, 277), and (3) release of toxic monocyte secretory products that serve as effector molecules in antimicrobial reactions such as H_2O_2, interleukin-1, or tumor necrosis factor alpha (274, 278-281). In agreement with these findings, the results in Chapter II of this dissertation demonstrate that HIV-infected myeloid cells retain the ability to produce superoxide and monocyte-specific enzymes. However, a variety of changes in monocytes from HIV-infected individuals have been reported including phenotypic marker expression, chemotaxis, and antigen-presenting function. While several flow cytometric analyses document normal phenotypic expression of plasma membrane antigens (MHC-II, CD4, CD11, CD14, CR3, Fc receptor I and II, or transferrin receptor) (278, 282, 283), others using similar methodologies report significant changes in the expression of these monocyte membrane antigens (284-286). Furthermore, several investigators report that AIDS patients have impaired Fc-dependent killing and others have shown that, although phagocytosis is normal, intracellular killing was deficient (287, 288). Monocyte chemotactic responses to several different chemoattractants are also depressed in HIV-infected individuals below that of uninfected individuals (277, 289-291). This observation

can be mimicked with monocytes from seronegative donors after exposure of the cells to purified gp41 or gp120 proteins, suggesting that defective chemotaxis is due to inhibiting factors present in the serum of HIV-infected patients analogous to the gp41 or gp120 proteins of HIV-1. In summary, derangement of hematopoietic cells and changes in mononuclear phagocytes of HIV-infected individuals are ambiguously defined and require further investigation.

The Role of Myeloid Cells in Production of Viral Variants.

Monocyte/macrophages and their precursor cells serve as intracellular reservoirs for virus and may contribute to the spread of HIV-1 to the peripheral tissues such as the skin, lungs, brain, and lymph nodes (199, 202, 226, 233, 234, 292-297). This is accomplished through a "Trojan horse mechanism", in which cells of monocytoid lineage conceal the viral genome by restricting virus replication. Initially, HIV-1 expression of infected HL-60 cells is low (Chapter II), similar to that seen *in vivo* for HIV-1 and other lentivirus systems (276, 298). *In vivo*, latently infected myeloid cells can multiply in the bone marrow and are subsequently released into the circulation where they undergo differentiation and maturation. Studies have shown the maturation of monocytes into macrophages relieves the restrictions of viral replication and promotes viral dissemination within target tissue or cells and blood monocytes of lentivirus infected animals (203, 275). Recent reports have shown that HIV-1 gene expression can be modulated by cytokines (47-50, 222-224). Therefore, the initial low level of viral expression seen *in vitro* in infected HL-60 cells, may be extended further *in vivo* by cytokines or other accessory cells. Although differentiation is not induced by HIV-1 infection of HL-60 cells, the low level of viral expression is overcome 15 days after infection and reaches levels seen in infected H9 cells (Chapter II). My studies suggest that during the course of HIV-1 infection of myeloid cells, selection for a specific genetic alteration occurs which allows the virus to efficiently replicate and spread in a cell in which it is normally dormant. This

variant, NL4-3(M), which is consistently isolated from infected HL-60 cells during the lytic phase of infection, allows efficient viral replication in HL-60 cells at an efficiency similar to what is seen in H9 cells (Chapter III). *In vivo*, this cytopathic variant could cause elimination of the pool of myeloid precursors reflected in the overall cytopenia observed in AIDS patients. Viral variants are known to arise throughout the course of HIV-1 infection (21, 112, 169-175) and recent evidence suggests that changes in the biological features of the virus with time (175, 177, 178) correlate with disease progression.

HIV-1 Replication in Myeloid Cells.

The genomic diversity of HIV-1 isolates is reflected in the biological properties of the virus including host range and cytopathicity. The host range of a virus is determined by three features: infectivity, rate of replication, and magnitude of infectious progeny. Infectivity relates to the ability of a virus to gain entry into target cells. After entry, infection is further characterized by the replication kinetics and magnitude or levels of virus production. Thus HIV-1 replication could be regulated both at the level of virus entry into a cell and during subsequent events. Defining the functional gene(s) of HIV-1 that controls these biologic properties in a given cell type could therefore help in the understanding of HIV-1 pathogenesis and in designing antiviral strategies. Recent evidence has implicated *env*, *vif*, *vpu*, *vpr*, and *nef* genes as contributing to HIV-1 replication and cytopathicity (13-16, 18-20, 103, 138, 160, 161, 163, 165-168).

HIV Genes Potentially Involved in the Restricted Growth of NL4-3 in Myeloid Cells.

The data presented in Chapter III and that of others (108) suggest that T cell tropic viruses are capable of infecting myeloid cells and macrophages; however, their replication in these cell types is restricted. The restricted growth of NL4-3 in HL-60 cells occurs prior to reverse transcription of the viral genome, as well as at a step after reverse

transcription. The envelope gene of NL4-3(M), the variant virus which is cytopathic to myeloid cells, differs in nucleotide sequence (Chapter IV) from the envelope gene of NL4-3. The importance of the envelope gene to HIV infection was demonstrated by replacing the envelope gene of NL4-3 with that of NL4-3(M) to generate a recombinant virus, NL4-3envA. NL4-3envA acquired the ability to productively infect monocyte-derived-macrophages. Although the change in host range is likely due to sequence alterations in the envelope gene of NL4-3, these differences lie outside the V3 loop (Chapter IV). This is contrary to early reports that macrophage versus T cell tropism was determined by sequences in the V3 loop (16, 18, 269). This data provides further evidence in support of the hypothesis that regions outside V3 loop contribute to viral tropism by influencing the conformation of envelope protein (174, 271). However, the altered envelope gene is not solely responsible for the biological phenotype observed for NL4-3(M), since NL4-3envA does not produce as much progeny virus from infected macrophages and does not cause the rapid cytopathic effect in HL-60 cells as seen with NL4-3(M) (Chapter IV). In addition to the *env* gene, the second exon of the *rev* gene is contain in the NL4-3envA. Since this encodes the majority of the Rev protein, it is unlikely that differences in the Rev protein account for differences in NL4-3(M) verses NL4-3 in HL-60 cells and macrophages.

The additional restriction(s) to growth of NL4-3 in HL-60 cells could be occurring at any of several regulatory steps in the viral life cycle. NL4-3 replication in HL-60 cells could be controlled by a mechanism similar to that of murine leukemia viruses in mice. *Fv*-1 is a normal mouse gene and alleles of this gene encode the ability to inhibit the replication of certain classes of murine leukemia viruses (MuLV's) in mice (313, 314). The two common alleles of *Fv*-1, *Fv*-1^{n} and *Fv*-1^{b} are so called because of their presence in the prototypical mouse strains NIH and BALB, respectively. *Fv*-1^{n} inhibits replication of B-tropic MuLV's, and *Fv*-1^{b} inhibits replication of N-tropic MuLV's. The determinants of viral tropism lie within the *gag*-encoded capsid (CA) protein and a swap

of two adjacent amino acids in CA between N and B sequences can completely reverse viral tropism (315, 316). Restriction is not absolute; usually between 10- and 1,000 fold fewer cells are productively infected in a restrictive host than in a permissive host (314). Studies of *Fv*-1 restriction in cell culture demonstrated that the restriction operates after entry of virus into cells but before or during integration of the viral DNA into the host cell genome (317-323). In some cases, restriction can be accounted for by an inhibition of viral DNA synthesis (323). In most cases, however, normal or nearly normal levels of linear viral DNA are synthesized in restricted cells, yet the level of integrated proviruses is severely decreased (317, 320, 321, 323). Likewise, viral DNA is detected in NL4-3 infected HL-60 cells with kinetics similar to that seen in NL4-3(M) infected HL-60 cells or NL4-3 infected H9 cells (Chapter III). However, with time there is a decrease in the level of NL4-3 DNA in the HL-60 culture suggesting that the viral infection is not spreading. The HIV-1 preintegration complex, isolated from nuclear and cytoplasmic extracts of $CD4^+$ cells after acute HIV-1 infection, contains viral RNA and DNA associated with viral integrase (IN), matrix (MA), and reverse transcriptase (RT) (324). A functional role for MA is suggested by the presence of several putative nuclear localization sequences which could influence nuclear import characteristics of the viral preintegration complex (324). If host cell factors present in HL-60 cells prevent the preintegration complex from being efficiently imported into the nucleus then integration of the viral DNA into the host genome cannot efficiently take place. Although integration may not be necessary for expression of HIV-1 protein products (230, 325-327), integration has been shown to be necessary for efficient gene expression and production of infectious virus (38, 326). Thus, host factors present in HL-60 cells could restrict import of the preintegration complex and/or integration of the viral DNA into the host genome. Since this restriction is not complete, it allows production of a variant virus, NL4-3(M), which overcomes the restrictions to replication of NL4-3 in HL-60 cells.

Another possible point of restriction to replication of NL4-3 in HL-60 cells is at the level of viral gene expression. Expression of HIV-1 genes exhibits complex regulation, involving both viral and cellular control elements (63, 328). One of the viral gene products which plays a central role in viral replication is the Tat protein. Tat, which binds to a stem-loop structure at the 5' end of the RNA termed the trans-activation response element (TAR), is a powerful trans-activator of gene expression at one or more control levels (60, 63, 328) and is required for efficient virus replication (58, 59). Tat directly interacts with nascent TAR RNA stem-loop *in vivo*, most probably binding to TAR in the form of a complex containing cellular cofactor(s) (329). Several investigators have demonstrated that formation of this RNA-protein complex can induce a significant increase in the level of mRNA synthesis from the adjacent long terminal repeat (LTR) promoter element of HIV-1, thus suggesting that TAR might be the RNA equivalent of a DNA enhancer element (330). Recently, it has been demonstrated that changes in basal promoter activity (331) and mutations in Tat (332) play a role in viral tropism. Since the activity of Tat relies on basal promoter activity and cellular cofactors (52, 53, 54, 333), changes in either Tat, TAR, and/or the LTR in NL4-3(M) compared to NL4-3 could account for the differences observed in the viral replication cycle in HL-60 cells.

An additional gene which could contribute to the restricted growth of NL4-3 in HL-60 cells is the viral gene *nef*. The Nef mRNA like Tat mRNA is detected early in infection (133). Once thought to have an inhibitory effect on the HIV-1 LTR and viral replication in cell culture (136, 137), recent evidence demonstrates that Nef has a positive role in efficient viral replication (138, 139) and in the development of AIDS in monkeys (140). Although the mechanism by which Nef acts is unknown, evidence suggests that Nef may be involved in signal transduction and viral and/or cellular gene regulation. Nef is a myristylated protein and possibly interacts with cell membranes and/or membrane associated proteins in a signal transduction cascade (135). In addition, Nef may act at a transcriptional level since it has been shown to interact with nuclear factors associated

with promoter elements, is capable of inducing down regulation of CD4 from the cell surface, and is capable of blocking induction of interleukin 2 mRNA (146-148). An alteration in the activity of Nef produced by NL4-3(M) may contribute to the increase in cytopathicity in HL-60 cells. Alternatively, changes in the interaction of Nef with the repertoire of cellular proteins available in HL-60 cells could account for the difference in the replication cycle of NL4-3(M) compared to NL4-3 in these myeloid cells.

One final point of regulation of viral replication is at the level of the viral particle assembly and release. The accessory genes *vpu*, *vif*, and *vpr* are thought to be involved in these final stages of the viral life cycle. The HIV-1 protein Vpu is an 81-amino acid amphipathic integral membrane protein with at least two known biological functions: (1) enhancement of virus particle release from the plasma membrane of infected cells and (2) degradation of CD4 in the endoplasmic reticulum (ER). In the absence of Vpu, an accumulation of intracellular viral proteins and cell-associated HIV-1 particles accompanied by increased cytopathicity were observed (115, 116, 117). In addition, it has been demonstrated that Vpu causes degradation of CD4 in the endoplasmic reticulum which results in enhanced intracellular transport and processing of the viral gp160 protein (119, 120, 121). More recently, investigators demonstrated that Vpu is phosphorylated in infected cells at two seryl residues by the ubiquitous casein kinase 2 (334). Although mutant Vpu lacking both phosphorylation sites was unable to degrade the HIV-1 receptor, CD4, in infected cells, and Vpu-mediated enhancement of virus secretion was only partially affected (335). This suggests that the two biological functions of Vpu are independent and exhibit a different sensitivity to phosphorylation.

Protein phosphorylation is known to be an important modification which is used to regulate cellular processes. Phosphorylation can either activate or inactivate the biological function of a protein, often in a reversible manner, such as cell cycle kinase cdc2 (336). Protein phosphorylation can also regulate such processes as : the initiation of protein synthesis as seen for eIF-2α (337), signal transduction as in the activation of NF-κB by

phosphorylation of its inhibitor IκB (338), the expression of cell surface receptors such as the phosphorylation dependent down regulation of CD4 (339), or the regulation of neurotransmitter function (340). During infection, the phosphorylation of the NL4-3 Vpu protein could be altered in HL-60 cells. The HIV-1 envelope polyprotein precursor, gp160, proceeds through the ER and Golgi complex and is proteolytically cleaved into the mature gp120 and gp41 components. Intracellular gp160-CD4 complexes can form in the Golgi complex preventing processing of the gp160 protein into gp120 and gp41 (119) which are elevated by phosphorylated Vpu protein (334). Thus, if Vpu is not properly phosphorylated in HL-60 cells then degradation of CD4 in the ER would not occur, impairing gp120 and gp41 production. This ultimately affects the assembly and release of virus particles and may contribute to the delay in the life cycle of NL4-3 in HL-60 cells. The inactivation of Vpu could be accomplished by the interaction of HL-60 specific host cell factors with Vpu preventing its phosphorylation, or alternatively, a lack of phosphorylation of Vpu in HL-60 cells. The variant virus NL4-3(M) may be capable of overcoming such a restriction and efficiently replicate in HL-60 cells. If so, Vpu phosphorylation in NL4-3(M) infected HL-60 cells could occur due to alterations in the phosphorylation sites of Vpu or to alterations in the Vpu protein which prevent interaction with an inhibitory factor in HL-60 cells.

The growth and host range of HIV-1 have also been shown to be influenced by Vpu in the context of the transmembrane glycoprotein, gp41 (165). Investigators have demonstrated that cloned HIV-1 isolates which contained a functional Vpu replicated poorly in the monocytic cell line THP-1 compared to the same isolate with a nonfunctional Vpu protein. However, in the T cell line H9 both viruses grew well. Thus, Vpu may affect the interaction of a cellular constituent with the viral envelope. Differential expression of a host cell factor could account for the effect observed in the different cell types and for the difference of replication of NL4-3 and NL4-3(M) in HL-60 cells.

Two other genes potentially involved in the restricted growth of NL4-3 in HL-60 cells are *vif* and *vpr*. By an unknown mechanism, Vif protein has been reported to increase the infectivity of progeny virus (107, 110) by as much as 100- to 1000-fold (100). Recent evidence suggests that Vif may also enhance cell to cell transmission of HIV-1 (101, 103, 107). Studies using Vif^{+} and Vif^{-} viruses demonstrate that Vif protein was required at the time of virus production (110). Together with the findings that vif mRNA is expressed late in infection (49, 299) and is present in infected cells but not virions (97, 101, 300, 301), these results indicate that the Vif protein acts late in the viral life cycle during the processing of virion proteins, virion assembly, or virion maturation. More recently, Vif has also been implicated as acting early in the viral life cycle. In T cell lines in which Vif function is indispensable for virion production, Vif was found to be necessary for efficient viral DNA synthesis (108, 160). In NL4-3 infected HL-60 cells, the activity of Vif could be hindered through interaction with myeloid specific cellular proteins. This lack of Vif function may account for the decrease in the levels of newly reverse transcribed DNA observed early after infection of HL-60 cells (Chapter III). Alternatively, the infectivity of the progeny virus produced within the first 15 days after NL4-3 infection of HL-60 cells could be low due to the inhibition of Vif function. This hypothesis is supported by the finding that virions released 11 days after infection of HL-60 cells with a T lymphotropic virus appeared either empty, containing several cores, or containing less envelope protein on their surface than the input virus or virions released 30 days after infection of HL-60 cells (262).

Vpr may also play a role in the replication phenotypes of NL4-3 and NL4-3(M) in HL-60 cells. Vpr protein is found in the cell nucleus, as well as in virions, suggesting that Vpr can act either early in the viral infection or late during particle assembly and maturation, and that Vpr may interact with cellular regulatory mechanisms important in the establishment of infection (123, 127, 129, 302). Studies have shown that Vpr might have a role in the upregulation of HIV-1 expression (129). The inability to identify

specific sequences in the HIV-LTR, which mediate the observed increase in virus expression, raises the possibility that Vpr may function in a manner similar to the herpes simplex virus VP16 protein (303, 304). VP16 is known to work in concert with a cellular factor to upregulate the expression of immediate early HSV genes (305). Previous studies have shown that Vpr is not required for HIV-1 infection or replication in $CD4^{+}$ lymphocytic cell lines *in vitro*, although inactivation of the protein leads to slower replication kinetics and delayed cytopathicity in these cells (105, 123, 130). On the other hand, Vpr has been shown to play a role in the regulation of virus replication in primary monocytes, and together with Vpu, mediate the expression of silent versus productive infection (306). In addition, Vpr has been shown to influence cellular differentiation (307). Through interaction with myeloid specific proteins, Vpr activity could be restricted in NL4-3 infected HL-60 cells. Thus alterations in Vpr, Vif, and/or Vpu may be involved in the generation of NL4-3(M) and consequently in overcoming the restrictions in the life cycle of NL4-3 in HL-60 cells.

Conclusion.

In conclusion, I have presented evidence in this dissertation that HIV-1 infection of myeloid cells has a distinct role in HIV-induced pathogenesis. I have shown that T lymphotropic viruses can infect myeloid cells resulting in delayed viral replication. The existence of poorly replicative HIV-1 isolates may be essential for establishing persistent myeloid cell/macrophage infection during the early, asymptomatic stage of disease. However, with time, viral variants emerge which can replicate efficiently in myeloid cells/macrophages. In my system, the variant NL4-3(M) was consistently isolated during the lytic phase of infection of myeloid cells. The stable genomic change(s) in NL4-3(M) allows efficient viral replication and increased cytopathicity in HL-60 cells, and extends the cell tropism to monocyte-derived-macrophages. It is likely that the envelope gene is involved in this altered phenotype but at least one additional alteration is required to

confer all of the biological properties of NL4-3(M). The onset of increased virus replication has been correlated with the onset of clinical disease. Thus the generation of variants like NL4-3(M) *in vivo* could have profound effects on the hematopoietic and immune systems, leading to the cytopenia and decreased immune function observed in AIDS patients.

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