VIROLOGIC STUDIES IN PEDIATRIC HIV-1 INFECTION

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Abstract

The proportion of HIV-1-infected children is increasing in parallel with that of women of child-bearing age. There are currently over 200 HIV-1-infected Canadian children. This study was undertaken with the main objective being to evaluate several clinical and laboratory measures in this population to gain a better understanding of their disease status and their risk for progressing to AIDS. These measures included viral load testing, drug resistance testing, CD4+ T cell levels, and viral phenotype testing. It was hypothesized that a group of clinical and/or laboratory tests exist which would be optimal markers by which to assess a pediatric HIV-1 patient's current status as well as the risk of disease progression over time. Secondary objectives included to determine the prevalence of resistance to zidovudine (ZDV) in HIV-1-infected Canadian children and to determine the association of drug resistance with the other clinical and laboratory variables evaluated.

HIV-1-infected pediatric patients were recruited from seven healthcare centers across Canada. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples donated by these patients. Plasma viral load determinations were performed. PBMCs were cocultured to generate viral stocks from which viral phenotype and standardized ZDV resistance testing assays were performed. PBMCs were also used to set up quantitative micrococultures which were used as the basis for our rapid phenotypic resistance assay and for determining cell-associated viral loads.

Data was available for a total of 86 patients. 23/78 (30%) HIV-1 positive viral isolates were of the SI phenotype. 14/60 (23%) isolates showed resistance to ZDV according to the rapid phenotypic resistance assay. Median cell-associated viral load was 125 IU/10^6 cells and median plasma viral load was 25 000 copies/ml. 26/53 (49%) isolates showed resistance to ZDV according to the genotypic resistance assay. A positive correlation was observed between cell-associated and plasma viral load ($r=0.37$, $p=0.004$). Plasma viral load and the presence of encephalopathy were also highly correlated ($p=0.023$), as were plasma viral load and perinatal transmission ($p=0.004$). In contrast, cell-associated viral load tended to be higher in children without encephalopathy and who had acquired HIV-1 non-perinatally (highly significant ($p=0.001$)). 35/45 (78%) of the samples on which comparative analysis of genotypic and phenotypic

resistance was performed produced concordant results. Phenotypic resistance, genotypic resistance, and SI phenotype were found to be correlated with lower CD4 cell counts and percents.

According to the results obtained in this study, the prevalence of ZDV resistance among HIV-1-infected Canadian children is high. It is surprising that more significant associations between different parameters were not found in establishing this "viral inventory". Associations may become more clear as prospective data are obtained.

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INTRODUCTION

Epidemiology of Pediatric HIV-1 Infection

The spread of HIV-1 throughout the world could be thought of as the sum of several epidemics in different populations. These populations include men having sex with men, intravenous drug users, and heterosexual men and women to name the major groups, most of which include women and children. The dynamics of these epidemics vary from continent to continent, country to country and even within different regions of a given country. However, none are discrete and they are all in a dynamic equilibrium with one another.

Neither are they discriminatory, as HIV-1 can infect anyone. The first cases of AIDS in children were reported in 1982 (1-3), one year after the first case was described in adults. In the nineteen years since then, HIV-1 infection has had a significant impact on the health of children worldwide. In 1983, HIV-1 was determined to be the cause of the syndrome. Since then, the growth of the AIDS epidemic in children has paralleled that of infection in women, with more than 70% of female cases of AIDS diagnosed during the childbearing years (13-39 years) (10).

As of November 20, 1996, the number of cumulative AIDS cases in adults and children reported to the World Health Organization since the onset of the pandemic was 1,544,067(4) and by December 1998, the total number of AIDS deaths since the beginning of the pandemic was 13.9 million (330). By the end of 1998 the World Health Organization had estimated that 13.8 million women and 1.2 million children were infected with HIV-1 worldwide (330). In total, it is estimated that the number of persons living with HIV-1/AIDS (including all asymptomatic individuals) at the end of 2000 was 36.1 million. Today, almost all children (85% in the United States and the vast majority worldwide) who acquire HIV-1 do so from their infected mothers, and this is termed vertical transmission (8). By the end of 2000, the World Health Organization had determined that 13.3 million women and children had died from HIV-1-related disease, and that 16.4 million women and1.4 million children were living with HIV/AIDS (73).

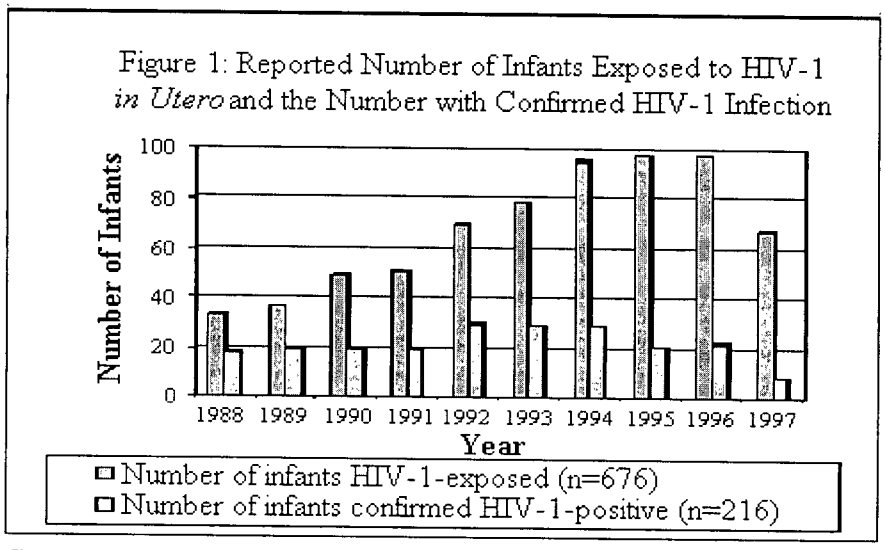
Originally, HIV-1 disease in North America was believed to be confined to men having sex with men. The spread of HIV-1 by heterosexual contact and intravenous drug use has increased the prevalence of

infection among sexually active women of childbearing age. Epidemiological surveys in Europe and North America show that of women delivering infants between 1989-1996, 0.15-0.24% were infected with HIV-1. In sub-Saharan Africa, the proportion of pregnant women receiving antenatal care who are infected is as high as 20-30% (74).

Many epidemiological studies have attempted to evaluate the rate of vertical transmission of HIV-1 infection. In Africa, reported transmission rates have ranged from 28-52% of infants born to infected mothers (62). In Europe and North America, transmission rates have been lower, ranging from 10 to 39% (63,67,109,110).

Epidemiology of Pediatric HIV-1 Infection in Canada

The first AIDS case diagnosed in Canada was in 1982 (331). By December 4, 1986, the total number of AIDS cases reported to the Laboratory Centre for Disease Control (LCDC) at Health Canada was 830 and 7690 by the end of 1991. Up to the end of December 1999, a cumulative number of 16 913 AIDS cases in Canada have been reported to LCDC; 196 of those cases were among children less than 15 years old. The total number of positive HIV tests among Canadian children under the age of 15 between November 1985 and December 1999 was 664. A Health Canada surveillance report published in April of 1999 reported that between 1984 and 1998, there had been 218 confirmed cases of pediatric HIV-1 infection in Canada, and 924 perinatally HIV-1-exposed infants (5). Figure 1 depicts the reported number of infants born to HIV-1 positive mothers and the number of infants with confirmed HIV-1 infection (1988-1997) (332).



Source: Bureau of HIV/AIDS, STD, and TB, LCDC, Health Canada, May 1999.

Table 1 shows the cumulative number of Canadian perinatally HIV-1-exposed infants, by geographic region and current status, 1984-1998 (5).

Table 1: Cumulative Number of Canadian Perinatal HIV-1-Exposed Infants

	Current Status up to December 1998							Total	
Region	Exposed Indeterminate[1]	Confirmed Infected Asymptomatic	Confirmed Infected Symptomatic[2]	Sero-reverted	Died of AIDS	Died/ Other	Unknown	n	%
British Columbia	3	1	32	102	6	2	0	146	15.8
Alberta	7	0	10	18	1	1	0	37	4.0
Prairie/ Territories[3]	12	0	13	48	4	0	0	114	8.3
Ontario	23	18	51	138	33	5	3	271	29.3
Quebec	17	26	63	198	59	9	1	373	40.4
Atlantic Provinces[4]	3	0	4	9	4	0	0	20	2.2
Total	**65**	**45**	**173**	**513**	**107**	**17**	**4**	**924**	**100.0**
%	**7.0**	**4.9**	**18.7**	**55.5**	**11.6**	**1.8**	**0.4**	**100.0**	**-**

[1]Maternal status was confirmed HIV positive but infant status was indeterminate at presentation.
[2]Includes mild, moderate, and severe combined.
[3]Includes Saskatchewan, Manitoba, and Northwest Territories.
[4]Includes Newfoundland, and Nova Scotia.
Source: Health Canada. *HIV and AIDS in Canada: Surveillance report to December 31, 1998.* Division of HIV/AIDS surveillance, Bureau of HIV/AIDS, STD, and TB, LCDC, HPB, Health Canada, April 1999.

The majority of perinatally infected children in Canada acquire HIV-1 from mothers who are either injection drug users or sexual partners of injection drug users (9). A startling trend in Canada is that the proportion of AIDS cases in women is increasing. Up to 1990, 6.2% of AIDS cases in Canada occurred in women. Between 1990 and 1995, this rose to 6.9%, and in 1996, it was 9.5%. As this proportion increases, so may the proportion of infants who become perinatally infected. While the majority of AIDS cases in women are still related to heterosexual transmission, the contribution of injection drug users to the total is rising (6.5% before 1990, 19.5% between 1990 and 1995, and 25% in 1996) (6).

Pathogenesis of Pediatric HIV-1 Infection

Human immunodeficiency virus type 1 (HIV-1), the virus which causes the majority of cases of acquired immune deficiency syndrome (AIDS), is a nononcogenic, cytopathic retrovirus. It belongs to the lentivirus family. Being an RNA virus, HIV-1 possesses the ability to synthesize a DNA provirus from a template of genomic viral RNA. It accomplishes this using a virally encoded DNA polymerase called reverse transcriptase.

Figure 2: HIV-1

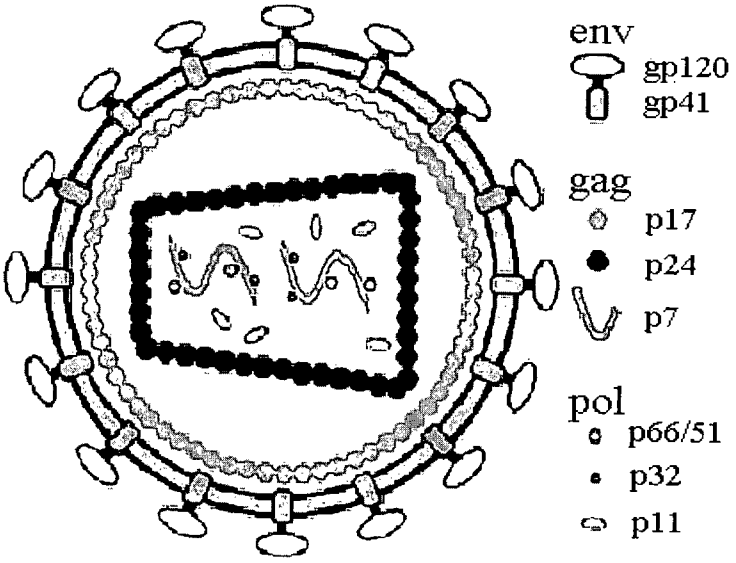


Figure 2 shows a schematic diagram of HIV-1. A mature HIV-1 virion is slightly more than 100 nm in diameter. When viewed by electron microscopy, it appears as a dense cylindrical core surrounded by a lipid envelope. The virion core contains structural proteins, the RNA genome and virally encoded enzymes. These enzymes include reverse transcriptase and integrase, which are needed in the early stages of viral replication.

The RNA genome of HIV-1 is ~10 kilobase (kb) pairs long and is comprised of the *gag, pol,* and *env* genes characteristic of most retroviruses, as well as two flanking long terminal repeat (LTR) sequences. It also contains at least six additional genes (*tat, rev, nef, vpu, vpr, vif*), which are needed for the coordination of viral gene expression and replication. Reverse transcriptase, protease and integrase are encoded by the *pol* gene, while the structural proteins of the virion core are encoded by the *gag* gene. The external and transmembrane envelope glycoproteins are encoded by the *env* gene.

Early in the AIDS epidemic, a dramatic decline in the absolute number of circulating CD4+ T cells and in the ratio of CD4+ T to CD8+ T cells was observed in adult AIDS patients (25, 26). The isolation of HIV-1 from the CD4+ T lymphocytes in the circulation of patients with AIDS (27-29), prompted investigators to propose the CD4+ T cell as the principal target of HIV-1 infection *in vivo*. HIV-1 was subsequently shown to infect and replicate in CD4+ T cells in culture and to cause a rapid and profound cytopathic effect in

these cells (30). The CD4 molecule on the surface of the CD4+ T cells is the high-affinity receptor by which HIV-1 preferentially infects the cells (31,32).

The life cycle of HIV-1 begins when it comes into contact with and binds to a CD4 molecule on a CD4+ T cell. This binding is mediated by the gp120 surface glycoprotein of HIV-1, a cleavage product of the gp160 precursor protein, the transmembrane glycoprotein (gp41) being the other cleavage product. As either a trimer or a tetramer of gp120/gp41, these proteins are incorporated into the outer lipid bilayer of the virion. Several specific sites on the gp120 molecule have been determined to be associated with CD4 binding. The affinity of gp120 for CD4 is greater than that of the MHC class II molecule (31,32).

The binding of the gp120 and the CD4 molecule triggers a series of events which results in the fusion of the virus and cell membranes at least partially mediated by the binding of a specific domain of gp120 to a chemokine receptor. Cocchi et al. demonstrated that the beta-chemokines, RANTES, MIP-1alpha, and MIP-1beta suppressed the replication of primary clinical isolates of HIV-1 *in vitro* (339), which led to the identification of chemokine receptors as HIV-1 coreceptors. Cellular chemokine receptors are seven-transmembrane, G-protein-coupled receptors, which transduce chemokine binding into intracellular signals. All HIV-1 strains use either the CCR5 or CXCR4 coreceptors; some use both. Sequences within the third variable region (V3) of the HIV-1 envelope gp120 seem to influence coreceptor usage.

The virus nucleocapsid core is then introduced into the host cell cytoplasm, and reverse transcription occurs, the genomic viral RNA being converted to proviral DNA. This is accomplished through the coordinated activities of the HIV-1 reverse transcriptase and a second virally encoded enzyme, ribonuclease H, which serves to degrade the viral RNA template. A second strand of DNA is then synthesized, producing a double-stranded DNA copy of the original HIV-1 RNA genome. The newly synthesized linear viral DNA is then translocated into the cell's nucleus, where it is integrated into the host's DNA, using the virally encoded integrase enzyme. In resting cells, the newly synthesized HIV-1 DNA may remain in an unintegrated circular form in the cytoplasm, until it is degraded or translocated to the nucleus following host cell activation. Once integrated, the proviral DNA may remain latent or be immediately transcribed into new viral RNA and proteins, through a complex interaction of host cell derived and virally-mediated events. A detailed discussion of these events may be found in Biology and Molecular Biology of HIV, by Ruth I. Connor and David D. Ho (333).

Clinical Course of Pediatric HIV-1 Infection

"The common denominator of infection with HIV-1 in children and adults is a profound immunosuppression, rendering the host susceptible to the development of various opportunistic infections and neoplasms (7)." Many of the clinical manifestations of HIV-1 disease are similar in adults and children. However, HIV-1 infection has other effects, which may be more profound in or confined to infants and children as organ systems such as the central nervous system are still not yet fully developed. The course of infection *in vivo* is controlled by complex interactions between the virus and the host's immune system. Following initial infection with HIV-1, many individuals experience a period of clinical latency, an asymptomatic phase with may last up to 10 years or more (334).

In contrast to older children and adults, infants infected with HIV-1 *in utero* or during the perinatal period (during mid to late gestation or within 28 days of birth) have a relatively short asymptomatic interval before they develop symptomatic disease. This being said, two distinct clinical courses are recognized. About one fifth of maternally infected children have a rapid decline in CD4+ T lymphocytes, resulting in an early diagnosis of AIDS and death, whereas most have a form of disease which progresses more slowly and is probably similar to that observed in adults (75,45,46,85,24).

Retrospective studies of perinatally infected children suggest that the median time from infection to the onset of clinical symptoms was 5-10 months (9,23,24), and that the mean incubation from birth to AIDS has been estimated as 4.1 months for rapid progressors and 6.1 years for slow progressors (46). The longest asymptomatic period reported in these studies was 7.3 years. It has been estimated that ~20% of HIV-1-infected infants will develop AIDS during the first year of life (46), and >90% of infected infants can be expected to develop HIV-1-related symptoms by age 12-18 months (67,75,24).

Defining the potential factors which contribute to long-term survival in children can assist in understanding the mechanisms of HIV-1 disease in this population. A number of anecdotal reports or studies with small data sets have been published (38,49,220). In a larger study of 182 children who survived longer than 5 years, De Martino and the Italian Registry for HIV-1 Infection in Children found that a substantial number of children do survive after early childhood and that severe disease, low CD4+ T cell count and p24 antigenemia do not necessarily preclude such long-term survival (221).

The exact reason for the bimodal distribution of disease progression remains controversial, although it has been suggested that it may be associated with the timing of infection during pregnancy and delivery. Blanche et al has shown that in maternally HIV-1-infected infants, the rate of disease progression varies directly with the severity of the disease in the mother at the time of delivery (227). It has also been hypothesized that HIV-1 infection may progress at different rates based on the tissue distribution and maturity of CD4+ T cells at the time of infection, specifically, disease may progress more rapidly in infants if the timing of transmission *in utero* coincides with the period of rapid expansion of CD4 immunocompetent cells in the fetus (41). This expansion would allow for the spread of HIV-1 into target cells, whose normal migration between the bone marrow, spleen, and thymus would allow for HIV-1 to diffuse throughout the body. The fact that the immune system in the infant is immature may also hinder its ability to respond effectively to restrict the spread of the virus. Thus, activation of HIV-1 infection in the perinatally infected infant could result in a much more rapid and severe destruction of immune function. Infection of immature target cells, such as noncirculating thymocytes or hematopoietic progenitor cells, may initially restrict levels of HIV-1 in tissues and peripheral circulation (21). However, if immunologically naïve cells are exposed to antigens or immune cytokines in the first few months of life, this may activate those cells, which would in turn increase virus replication, thereby adding to the virus burden in plasma and circulating mononuclear cells (22). Additional factors, including the strain of virus passed from mother to infant, may also contribute to viral pathogenesis and disease progression in infected neonates and children.

The hallmark of immune deficiency in pediatric HIV-1 infection, as in adult infection, is the progressive decrease of CD4+ T lymphocytes. However, a simple quantitative decrease of this cell population does not sufficiently explain all irregularities in immune responsiveness known in pediatric HIV-1 infection. Almost immediately following infection in adults (independent of the route of infection), a continual loss of CD4+ T lymphocytes usually in conjunction with a rise in CD8+ T lymphocytes is seen, resulting in the reversal of the ratio of CD4+ to CD8+ T cells in the peripheral blood. The most reliable predictor of clinically relevant immune deficiency is the CD4+ T lymphocyte count itself, with most opportunistic infections occurring when it decreases below 200-300 cells/mm^3.

Neonatal HIV-1 infection is also characterized by a continuous decline in CD4+ T cells (1,2,42-44). Newborns have a much higher percentage of circulating CD4+ T lymphocytes than adults and also a higher percentage of naïve compared with memory T lymphocytes. This persists over the first two years of life (132). Thereafter, the percentage and absolute numbers of CD4+ T cells in infants begin to approximate those of adults. The rate of CD4+ T cell decline is often much more rapid in neonatal infection than in adults, with the expected reversal of the CD4/CD8 ratio.

As in adult infection, CD4+ T cell reduction in neonates correlates with clinical disease as demonstrated by opportunistic infections and other symptoms. In multiple studies, loss of CD4+ T cells or reversal of the CD4/CD8 ratio has correlated with circulating viral load, loss of mitogenic responsiveness, and development of opportunistic infections, with the exception that opportunistic infections occur in HIV-1-infected neonates at much higher CD4+ T cell counts than in HIV-1-infected adults. In one study, 8 of 22 infants diagnosed with *Pneumocystis carinii* pneumonia had >450 CD4+ T cells /μl (47).

B cell abnormalities have a major impact on immune deficiency in pediatric HIV-1 infection, whether directly or as a consequence of CD4+ T cell dysfunction. Increased absolute numbers of circulating B cells are present in HIV-1-infected infants. Hypergammaglobulinemia, especially of the IgG or IgA class, is commonly the earliest laboratory abnormality of HIV-1 infection and is normally detectable by the age of 6 months (69,75). It is often used as an indicator of HIV-1 infection during the first few months of life. Levels of IgG increase with the age of the child and also with the stage of HIV-1 infection, the highest levels being present in older children and when infection is symptomatic (44). Abnormalities in B cell function cause susceptibility to bacterial infections, particularly those caused by *Streptococcus pneumoniae*, *H. influenzae*, Salmonella species, *Staphylococcus aureus* and *Escherichia coli*. Repeated infections caused by these agents are considered AIDS-defining illnesses in children (48). Other related immunologic abnormalities include decreased serum levels of insulin-like growth factor-1 and growth hormone (49), or increased levels of β_2 microglobulin and neopterin.

The most common and specific laboratory findings associated with HIV-1 infection in infants are a decreased CD4+ T cell count, an inverted CD4/CD8+ T cell ratio, and polyclonal hypergammaglobulinemia. These findings are not only highly indicative of HIV-1 infection but also give us a quantitative measure of immune deficiency. Any of these findings along with any unexplained

marrow dysfunction (anemia, leukopenia, thrombocytopenia) should prompt further investigations to rule out HIV-1 infection.

Many physical findings are associated with HIV-1 infection, mostly as a result of secondary opportunistic infections and malignancies. Diffuse lymphadenopathy is the most common condition directly related to HIV-1 itself. Hepatosplenomegaly, parotid enlargement, and tonsillar hypertrophy may relate to this chronic lymphoid stimulation. In one prospective study, at least one of these findings was present in 73% of HIV-1-infected infants before their first birthday (75). Parotitis, due to lymphocytic infiltration of the parotids is present in 10-43% of cases (130).

Early symptoms of HIV-1 infection may be nonspecific, but their persistence and severity should raise suspicion (131). A common early presentation is recurrent oral candidiasis with failure to thrive.

HIV-1 infection of the central nervous system (CNS) causes several distinct neurological syndromes, including subacute encephalitis, vacuolar myelopathy, aseptic meningitis, and peripheral neuropathy. Significant neurodevelopmental changes also occur, with failure to reach specific milestones often noted very early on (49). It has been shown that macrophages within the brain and CNS are primary targets for HIV-1 infection. Detection of HIV-1 DNA in brain tissue (222,223) as well as the isolation of infectious virus from the brain and cerebrospinal fluid (13,14) strongly suggests that HIV-1 may be a causal factor in the subacute encephalitis associated with AIDS.

Complications of HIV-1 infection can be classified according to their etiology. Opportunistic infections are the primary sources of morbidity and mortality in HIV-1-infected children. Fortunately, many of these infections can be prevented or effectively treated.

Bacterial Infections

HIV-1-infected children are at risk for various types of bacterial infections. This may be caused by cellular immunodeficiency (*Listeria monocytogenes, Salmonella* species), neutropenia, frequent use of indwelling devices (*Staphylococcus* species, *Pseudomonas* species), and inadequate humoral responses to encapsulated organisms such as of *Streptococcus pneumoniae* and *Haemophilus influenzae*. Serious recurrent bacterial infections (two or more in a 2-year-span) constitute an AIDS-defining illness in children (224). Serious infections, which occur more frequently in HIV-1-infected children, as compared with uninfected children, include pneumonia, bacteremia, sinusitis, meningitis, urinary tract infection, and soft

tissue infection (133,48,134). Most of these infections are caused by common childhood organisms, particularly *S. pneumoniae* (133). Gram-negative infections are frequently terminal, and often follow prior hospitalization and extensive antibiotic therapy (131).

The use of intravenous gammaglobulin (IVIG) for the prevention of bacterial infections has been controversial, mainly due to the lack of adequately controlled studies. A multicentre, randomized, double-blind, placebo-controlled trial of the efficacy of monthly IVIG versus albumin (placebo) infusions in 372 symptomatic HIV-1-infected children showed that IVIG was effective in prolonging the time to the development of a serious bacterial infection, and decreased the number of serious bacterial infections and the number of hospital admissions for children with CD4+ T lymphocyte counts of at least 200 cells/mm^3 (133). Two other trials have achieved similar results (225,226), although survival rates were unchanged. Overall, the use of IVIG is only recommended in selected groups of HIV-1-infected children.

Mycobacterial disease is becoming increasingly important in HIV-1-infected children. Infections due to *Mycobacterium avium* complex (MAC) occur in severely immunocompromised patients (i.e. low CD4+ T lymphocyte counts). These organisms (*M. avium, M. intracellulare*) are found widely in the environment. Infections are usually disseminated and fever, diarrhea, and wasting are the most common manifestations. Wasting is the most serious problem and thought to be the most important factor which causes the life expectancy in patients with HIV-1 and MAC infections to be shortened (135). The organism may be isolated from feces, blood, bone marrow, sputum, and gastric lavage fluid. Improvement in many patients can be achieved with multidrug regimens (three or more drugs) if given for prolonged periods of time as early as possible in the course of the disease (135). The effect of treatment on survival is not known. Problems which are difficult to control with therapy are diarrhea, weight loss, and anemia. Agents used in the treatment of MAC infections are rifampin, amikacin, ethambutol, ciprofloxacin, and clofazimine (an agent commonly used for *Mycobacterium leprae* infections), and less commonly, cycloserine and ethionamide.

Viral Infections

Most viral pathogens reported in HIV-1-infected children are members of the herpes-virus family. Table 2 shows the most common viral pathogens in HIV-1-infected children and their clinical presentations (10).

Cytomegalovirus (CMV) infection occurs commonly and can affect life expectancy (140). The most important clinical manifestations associated with CMV infection are hepatitis, pneumonitis, esophagitis, encephalopathy, retinitis, and colitis. Dissemination with multiorgan involvement can occur. CMV colitis with and without hemorrhage in both HIV-1-infected adults and infants has been described (141). CMV encephalitis in infants with HIV-1 infection has been documented. However, the diagnosis of CMV encephalitis can be difficult because of concurrent HIV-1 involvement of the central nervous system. CMV retinitis, common in adults, occurs less frequently in HIV-1-infected children (142). Treatment with ganciclovir is recommended, especially in patients with localized disease (i.e. retinitis, colitis), but renal and liver function and hematologic parameters should be monitored in light of known drug toxicity. Concomitant treatment with zidovudine could be hazardous due to potential synergistic bone marrow toxicity.

Table 2: Common Viral Pathogens in HIV-1-Infected Children

Viral Agent	Clinical Presentations
Cytomegalovirus (CMV)	Hepatitis, pneumonitis, retinitis, colitis, esophagitis, encephalitis
Herpes simplex virus (HSV) types 1 and 2	Recurrent or progressive mucocutaneous disease, may disseminate
Varicella-zoster virus	May become progressive, recurrent, or chronic with hyperkeratotic lesions Zoster: may appear shortly after varicella
Epstein-Barr virus (EBV)	Lymphocytic interstitial pneumonitis (?), parotitis (?), lymphomas, hairy leukoplakia
Respiratory syncytial virus (RSV)	Higher incidence of pneumonitis, wheezing uncommon
Measles	Interstitial pneumonitis, rash may be absent
Molluscum contagiosum virus	Aggressive and longer clinical course, larger lesions
Papillomavirus	Higher prevalence of large condyloma acuminatum lesions, recurrence common

Herpes simplex virus (HSV) also affects HIV-1-infected children and usually presents as recurrent episodes of gingivostomatitis or perineal ulcerations (3). These infections can become chronic and at times disfiguring. Treatment with topical, oral, or intravenous antivirals such as acyclovir is often necessary to control the infection. Severe primary chickenpox (caused by varicella zoster virus or VZV) or recurrent infection in a dermatomal distribution may be seen in some children. Recurrences may happen shortly (weeks/months) after varicella (143). As these represent an unusual complication in young children, their presence can be considered a potential indicator of immunodeficiency. Zoster infection may become

chronic and be associated with hyperkeratotic changes (143,144). Because of the risk of serious disease, the use of varicella zoster immune globulin (VZIG) is indicated in HIV-1-infected children exposed to active cases of chickenpox. In treatment of active disease acyclovir remains the drug of choice for HSV and VZV infections, but drug resistance is reported in HIV-1-infected adults. In these cases, foscarnet could be used since some favorable responses to this drug in these circumstances have been reported (145,146). Experience with foscarnet in children is limited. Vidarabine has not been shown to be effective in the treatment of herpetic infections in HIV-1-infected patients (145,146).

Epstein-Barr virus (EBV) infection in HIV-1-infected children is suspected to play a role in the pathogenesis of lymphoid interstitial pneumonitis (LIP) and certain lymphomas, though it's clinical manifestations are not thoroughly understood. EBV is also implicated in hairy leukoplakia, an oral lesion quite prevalent in HIV-1-infected adults but uncommon in children.

Increased morbidity and mortality from common respiratory viruses have been reported in children with HIV-1 infection. Symptoms associated with respiratory syncytial virus (RSV) infection include pneumonia, relative absence of wheezing, and prolonged viral carriage. Mortality may be higher (20%) than in non-infected children (147). If discovered early in patients with respiratory complaints, therapy with ribavirin might be instituted. Adenovirus and parainfluenza 3 virus may also have a more virulent course in these children (148,149).

Fatal measles has occurred in HIV-1-infected children (150). Children exposed to measles should receive IVIG although the efficacy of this treatment is unknown. Prevention of measles (even though it is a live vaccine) is essential and immunization is recommended even in symptomatic HIV-1-infected children.

Fungal Infections

Mucosal infections with *Candida* species occur at some time in the majority of children with HIV-1 infection. Oral candidiasis appears as pseudomembranous creamy plaques, as erythema on the hard palate or dorsum of the tongue (atrophic), or as an angular cheilitis. The plaques are easy to remove and an erythematous base remains. Cultures and biopsies are seldom required for diagnosis. These lesions usually respond to topical nystatin or clotrimazole troches. For patients who do not respond to the initial treatment, a short course of oral ketoconazole may be beneficial. Recurrence is common, especially if the patient is treated with antibacterial drugs, and may prove very difficult to control in some patients. These patients

may require chronic therapy. *Candida* esophagitis also occurs with or without concurrent oral involvement. Clinical symptoms of esophagitis include pain on swallowing, poor appetite, weight loss, and vomiting. *Candida* esophagitis usually responds rapidly to oral ketoconazole. Intravenous amphotericin B is administered when treatment with ketoconazole fails.

Disseminated candidiasis has been observed, usually in the hospitalized child as a complication of infected indwelling devices and antibiotic use (151). Secondary prophylaxis for thrush is frequently used. Most patients will do well treated with topical agents like nystatin (Mycostatin) suspension or clotrimazole troches. Some patients will require ketoconazole.

Infections with other fungal pathogens are uncommon in pediatric patients (151).

Pneumocystis carinii Pneumonia (PCP)

P. carinii is a well-known cause of pneumonia in malnourished infants and in immunocompromised hosts (136). PCP is the most frequent and lethal opportunistic infection in HIV-1-infected children under one year of age (136), representing 39% of AIDS-defining illness in children reported to the CDC through 1990. It is often the presenting illness associated with HIV-1 infection in infants (137,138). Most cases of PCP occur between 3 and 6 months of age, and 10% to 20% of HIV-1-infected infants may acquire it (231). Approximately one third of these infants die within 2 months of a PCP diagnosis (231). The fact that it is a primary infection as opposed to a reactivation infection, may explain the different pathophysiology compared to in adults.

Mortality from PCP is high in infants (often because the diagnosis is delayed, as HIV-1 infection may not have been previously suspected). In patients requiring respiratory support, acute respiratory distress syndrome is a frequent complication, further worsening the prognosis (139).

Trimethoprim-sulfamethoxazole combination (TMP-SMX) and pentamidine are the two most commonly used drugs to treat PCP. Due to its safety and efficacy profiles, TMP-SMX is the first-line drug for treatment in children and pentamidine is reserved for those patients who are hypersensitive to TMP-SMX or who are considered treatment failures.

There are few adequate data regarding the use of steroids as an adjuvant therapy for PCP in children. McLaughlin et al describe greatly improved survival in children infected with HIV-1 who have PCP-related respiratory failure when treated with adjunctive corticosteroids (228). One other previous study also

showed benefit (230). Unfortunately, no recommendations regarding the use of steroids in children younger than 12 years of age were made by the National Institutes of Health-University of California consensus panel on the use of corticosteroids as adjunctive therapy for *Pneumocystis carinii* in 1990 (229). Because of the high mortality associated with PCP and since both primary and secondary prophylaxis has been shown to be effective in other immunocompromised hosts (i.e. children with leukemia) and in adults with HIV-1 infection, guidelines for PCP prophylaxis in HIV-1-infected or -exposed children have since been developed (232), based on age-adjusted CD4+ T lymphocyte counts, to an equivalent of 200 cells/mm^3 in adults (132,137,138). Thea et al have published a study reporting evidence that primary antimicrobial PCP prophylaxis is highly effective in decreasing the frequency of PCP and early death in infants with perinatal HIV-1 infection (233)

Other Parasitic Infections

Parasitic pathogens seen in association with HIV-1 infection in children are numerous and varied. Congenital toxoplasmosis with CNS involvement has occurred in HIV-1-infected children whose mother probably acquired toxoplasmosis prior to conception (152-154). In immunosuppressed HIV-1-infected women, chronic parasitemia may occur more commonly, which places their children at risk for vertical transmission of toxoplasmosis. Congenital *Toxoplasma* encephalitis with discrete nodules or abscesses can occur. Congenital toxoplasmosis should be ruled out in HIV-1-infected young infants with neurologic involvement since it is amenable to therapy.

Congenital toxoplasmosis can be difficult to diagnose because of a weak or absent antibody response in the child. Treatment with pyrimethamine and sulfadiazine with folinic acid is the approach of choice when congenital toxoplasmosis is strongly suspected or documented. Toxoplasmosis in older children may have a clinical presentation similar to that in adults (i.e. *Toxoplasma* cerebral abscess). Infection in these cases will usually be caused by ingesting raw or poorly cooked meat or being exposed to cat feces. Therapy is the same as described above.

Diarrhea caused by the intestinal protozoans Cryptosporidium and *Isospora belli* is well recognized in HIV-1-infected patients. In immunocompetent individuals, cryptosporidiosis produces a self-limiting illness but in HIV-1-infected patients it can produce a severe secretory diarrhea with massive fluid losses, much like cholera. Treatment is mainly supportive. Maintenance of fluid and electrolyte balance and

central hyperalimentation are the most important. Therapy of cryptosporidiosis is much more problematic (155). Diarrhea due to *Isospora* usually responds well to TMP-SMX therapy.

Lymphoid Interstitial Pneumonitis

Lymphoid interstitial pneumonitis/pulmonary lymphoid hyperplasia complex (LIP) is one of the most common pulmonary conditions associated with HIV-1 infection in children (45,156) and is a criterion for diagnosis of AIDS in children less than 13 years old (224). LIP is not seen in adults. Its onset is usually insidious and may not be clinically evident in the early phases (3,156). Coughing is the most frequent symptom. Listening to the chest usually reveals normal findings but wheezing may be present. Clubbing occurs frequently, over time. The typical radiographic picture shows symmetric bilateral reticulonodular interstitial infiltrates and hilar adenopathy. With progression of disease, the nodules enlarge and coalesce. Histopathology usually shows a mononuclear interstitial infiltrate mostly composed of immunoblasts, plasma cells, and CD8+ T lymphocytes. A peribronchiolar component and lymphoid nodule formation are sometimes seen and may represent a more advanced stage of the disease (156). These lesions may remain static over long periods or progress to capillary block with arterial desaturation and chronic lung disease. In a small number of patients, LIP resolves spontaneously.

Definitive diagnosis is made by lung biopsy. Potential causes of interstitial infiltrates other than LIP, such as tuberculosis, PCP, and CMV infection should be excluded and a trial of antibiotics should be administered when the diagnosis of LIP is considered in a symptomatic patient. In those who lack respiratory symptoms, the diagnosis is based on radiographic findings alone. Bacterial infection is a frequent complication but PCP is seldom seen. The prognosis is usually good when LIP is the first symptom in patients with HIV-1 disease (45).

The pathogenesis of this disorder is not well understood. LIP is the pulmonary manifestation of a systemic process of immune activation, which includes parotid enlargement, generalized lymphadenopathy, and hypergammaglobulinemia as the most obvious manifestations (156). The management of most patients with LIP is limited to observation and monitoring of oxygen saturation. Some patients require supplemental oxygen intermittently (overnight) or on a continuous basis. Anecdotal reports of children with progressive LIP with oxygen desaturation treated with steroids suggest a beneficial effect.

Neoplasms

Malignancies have been reported to the CDC as the AIDS defining illness in about 2% of pediatric patients (197) and in a number of patients, it was the initial HIV-1-associated illness. CNS lymphoma is found in up to 6% of adult patients but only a few cases have been reported in HIV-1-infected children (157,176,178). Burkitt's Lymphoma/B-cell leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, and pulmonary leiomyosarcoma have all occurred in children (197-200). Kaposi's sarcoma is rarely clinically evident in children (201) and usually presents as diffuse lymphadenopathy.

The incidence of secondary malignancies in HIV-1-infected children will most likely increase since treatment with antiretroviral agents will prolong life, and because of the relentless increase in the number of new HIV-1 infections. Many patients respond well to chemotherapy, especially when the malignancy is the presenting illness and the overall condition of the patient is good (198,200). Certain tumors like CNS lymphomas may be radiosensitive (157,176). HIV-1 infection should not be a contraindication for chemotherapy and radiotherapy. Better policies for the management of these patients are needed, especially regarding eligibility for novel chemotherapy protocols, which often exclude patients with underlying immune disease.

Another way of reviewing the conditions associated with HIV-1 infection in children is according to the affected system, as the patient would present for medical evaluation.

Central Nervous System and Developmental Complications

CNS abnormalities are common and have been reported in 50% to 90% of children with HIV-1 infection (157-159). In children, the most important problem is the development of an encephalopathy, which may be the presenting feature of HIV-1 infection for some patients (160).

There is evidence suggesting that AIDS encephalopathy is a consequence of HIV-1 infection of the CNS (158,161,162,170). HIV-1 nucleotide sequences have been isolated from the brains of both adults and children with encephalopathy. HIV-1 has also been cultured from the cerebrospinal fluid (14). Intrathecal synthesis of specific HIV-1 antibody has also been demonstrated (158,162,169). At least one report has suggested that tumor necrosis factor may mediate myelin damage (163,171). The severity of encephalopathy in infants may be compounded by environmental considerations, including maternal use of illicit drugs during pregnancy.

Since HIV-1 infection occurs during the development of the CNS, the associated encephalopathy has some features which are unique to children. In particular, loss of developmental milestones, and diffuse bilateral pyramidal tract signs are reported. Acquired microcephaly, ataxia, seizures, and other movement disorders are also seen (168). Radiologic findings in HIV-1 encephalopathy include calcification of basal ganglia vessels, usually visible on computed tomography (CT) scan (164), and cerebral atrophy (on CT scan or magnetic resonance imaging (MRI) of the brain). Cerebrospinal fluid analysis may show mild pleocytosis or elevated protein levels.

Recent studies have documented that more subtle neurodevelopmental deficiencies may occur in asymptomatic and in mildly ill HIV-1-infected children. In one (165), children with transfusion-acquired HIV-1 infection during the neonatal period had diminished school achievement and performed poorly in tasks requiring motor speed, visual scanning, and cognitive abilities when compared to matched uninfected controls. In another (166), language capabilities at 18 to 30 months were impaired in asymptomatic HIV-1-infected children born to substance-abusing mothers, compared to matched uninfected controls. Although the pathogenesis of these abnormalities is unclear, HIV-1-infected children should be monitored routinely for neurodevelopmental deficits and referred for early intervention. Further studies are required to define the full spectrum of neurodevelopmental and cognitive defects in this population.

Finally, in a minority of cases, a more progressive encephalopathy has been described, with a median survival of less than one year (45). Pathologic findings described include corticospinal tract changes (167), demyelination with or without axonal damage (with evidence of HIV-1 in brain tissue), severe white matter degeneration and inflammatory cell infiltrates with macrophages, microglial nodules with and without multinucleated giant cells, and various forms of vascular disease (158,171). Therapy with zidovudine may stabilize or reverse the progression of this encephalopathy (172-174). In one study, brain growth, cognitive improvements, and improvement of auditory brainstem responses were observed (175).

Otorhinolaryngologic Complications

Otorhinolaryngologic problems are common in HIV-1-infected children, but seldom life-threatening. Otitis media and sinusitis are common complications and may be acute, recurrent or chronic. The prevalence of pathogens typifies that of immunocompetent hosts. In otitis media, pneumococcus, *Moraxella catharrhalis*, and *H. influenza* prevail. Initial treatment should be the same as that for an

immunocompetent host. Unfortunately, symptomatic children do appear to have more therapeutic failures despite adequate antimicrobial therapy (179). The child with recurrent ear infections should be considered for antibiotic prophylaxis or ventilation tubes, as appropriate. Chronic suppurative infections require aggressive drainage and broad-spectrum intravenous antibiotic therapy that includes coverage for *Pseudomonas*. Patients with sinusitis usually require 14 to 21 days of antibiotic therapy. Pneumonia may develop as a complication of recurrent sinusitis and should be borne in mind if appropriate symptoms develop.

Nearly all patients develop lymphadenopathy, especially in the cervical region. Lymph nodes are usually not tender, firm and freely moveable. When malignancy or opportunistic infections are suspected, a biopsy and/or aspiration are warranted. Otherwise, the nodes need only be observed. Bacterial adenitis occasionally occurs and is often found in association with LIP and elevated immunoglobulin levels. In these cases, the nodes enlarge quickly and are usually tender. This typically responds well to antibiotics. As described above, primary, recurrent, or chronic HSV infections of the oral cavity are also seen. These lesions can progress and affect adjacent areas and may require oral or intravenous acyclovir therapy. VZV may affect one or more branches of the trigeminal nerve, causing acute and severe pain.

Cardiovascular Complications

Cardiac complications associated with HIV-1 infection are increasingly recognized (182,183,188). These abnormalities are often minor and asymptomatic. In a prospective study of 88 symptomatic HIV-1-infected children who underwent electrocardiographic and echocardiographic evaluations as part of a zidovudine study, more than half had abnormalities but only 14% of patients required medical intervention for cardiac reasons (188).

The prevalence of cardiovascular abnormalities varies among different studies. With noninvasive methods, from 50% to more than 90% of HIV-1-infected children showed some kind of cardiovascular abnormality during the course of HIV-1 infection (182,184,188).

The most common findings from echocardiographs are ventricular dysfunction and pericardial effusion. Pericardial effusion is found in about one third of children (182,183). Obvious signs of cardiac dysfunction usually occur later in the disease (45). The presence of cardiac dysfunction may be masked by pre-existing clinical conditions such as hepatosplenomegaly, fever, pulmonary infections, and anemia

(182,183). Patients typically die of noncardiac causes (183), but deaths attributed to heart failure have been reported (182).

Pathologic examinations usually show enlarged hearts with hypertrophy and/or dilatation (182). Mononuclear infiltrates are seen in the myocardium (with or without necrosis) (182). Evidence of opportunistic pathogens has rarely been found (182,183,185). The HIV-1 genome has been isolated within the myocardium in adults and children using *in situ* hybridization (186,187), suggesting a direct role for the virus in cardiac disease. Other possible indirect mechanisms include autoimmunity and cytokine-mediated disease. There is no apparent association between the presence of cardiac abnormalities and immunologic abnormalities (188).

Patients with significant cardiac dysfunction usually respond well to medical intervention. Treatments include diuretics, digitalization, and fluid restriction and should be started when clinically significant cardiac dysfunction is diagnosed (183). Zidovudine given over a 6-month period does not appear to alter the course of cardiac disease (188). Anemia, hypoxemia, and nutritional deficiencies are potentially aggravating factors. The lack of adequate knowledge of the pathophysiology of cardiovascular disease limits capabilities to prevent or revert it in HIV-1-infected children.

Gastrointestinal and Nutritional Complications

The gastrointestinal tract is commonly involved in children infected with HIV-1. This frequently causes nutritional deficiencies and failure to thrive. Poor nutrition may be the result of insufficient caloric intake due to infectious complications of the oral cavity and esophagus, gastrointestinal malabsorption, and chronic or recurrent gastrointestinal or systemic infections.

Acute and chronic diarrhea in HIV-1-infected children are the most common presenting conditions, many of which are treatable, especially if a specific infectious etiology is identified.

Nephrologic Complications

Although data on renal disease in children with HIV-1 infection are limited, the condition does occur in this population (181). Based on published studies, 30-55% of HIV-1-infected children will have renal disease or urinary and electrolyte abnormalities at some time during the course of infection (234), and 3-29% will have more definite evidence of renal disease (181,234). In children, clinical manifestations include proteinuria, edema, intermittent hematuria, metabolic acidosis, and hyponatremia. Persistent

proteinuria is a common early finding. Clinically evident renal involvement is a late finding. In three studies, the mean age of onset of progressive renal disease was between 35 and 39 months (range, 13-90 months) (45,181,234).

Ultrasound examination usually shows enlarged and hyperechoic kidneys but these findings are non-specific and are similar to those of acute glomerulonephritis. Pathologic examination of kidney tissue obtained by percutaneous biopsy from children with clinically evident renal disease showed several types of lesions, including focal glomerulosclerosis, mesangial hyperplasia, focal necrotizing glomerulonephritis, and minimal-change disease (181). Renal failure was associated with focal glomerulosclerosis but was not considered to be the direct cause of death. Irrespective of the histology type and the level of renal function the outcome was poor once clinically evident nephropathy was diagnosed.

Some of the medications used to treat HIV-related conditions cause renal toxicity and this requires careful observation.

Results of specific management of end-stage renal disease are discouraging. There is minimal experience with dialysis in pediatric patients with HIV-1-infection. Experience with peritoneal dialysis is very scanty but may be preferable to hemodialysis. Generally speaking, these patients are very poor candidates for renal transplantation. In many instances, supportive management is the best that can be offered to patients with renal failure. The need for renal biopsy should be considered on an individual basis since specific therapy might be used in certain glomerulopathies.

Hematologic Complications

Anemia, leukopenia, and thrombocytopenia are common in the course of HIV-1 infection in children. Microcytic/hypochromic anemia is most common. This anemia may have an iron deficiency component, which may be treated with an oral iron supplement. Anemia frequently complicates zidovudine therapy in about 20% of children, and is typically macrocytotic. Autoimmune hemolytic anemia has also been described and is part of the spectrum of autoimmune phenomena affecting these patients. Treatment with steroids may elicit some response (189). Neutropenia is seen in about half of children undergoing zidovudine therapy and often requires dose reduction or temporary suspension of therapy (173). Thrombocytopenia occurs in about 13% of HIV-1-infected children (190) and may be the presenting condition (191). Significant bleeding may occur, including CNS hemorrhage. Bone marrow examination

usually uncovers normal to increased numbers of megakaryocytes and the majority of patients have increased levels of antiplatelet antibodies and circulating immune complexes. It appears that both shortened platelet life span and suppressed platelet production occur in HIV-1-infected patients. Zidovudine produces a temporary elevation in platelet counts in most HIV-1-infected adults (192-194), and children (195); however, many treatment failures in children have been reported (196). Some patients respond to high-dose IVIG infusions (190), but this treatment rarely results in a sustained remission. Many patients with clinically relevant thrombocytopenia will require steroids and eventually splenectomy (190) in order to achieve a sustained remission.

Clinical Interventions

Significant advances have been made in the use of antiretroviral agents in HIV-1-infected children, building on therapeutic advances made in adult medicine. In this context, the developmental impact of AIDS in children and the different clinical manifestations and altered pharmacokinetics of drugs in the pediatric population have been considered. Most of the new information comes from the use of the dideoxynucleosides zidovudine (3'-azido-3'-deoxythymidine, ZDV, azidothymidine, AZT, Retrovir), 2',3'-dideoxyinosine (didanosine, ddI, Videx), and 2',3'-dideoxycytidine (ddC, Zalcitabine).

Zidovudine

The first effective drug made available for the treatment of AIDS was the dideoxynucleoside zidovudine (ZDV). It is a thymidine analogue whose principal mode of action is to block HIV-1 replication (235,237). ZDV is phosphorylated by cellular enzymes into a 5'-triphosphate form which interferes with the viral RNA-dependent DNA polymerase reverse transcriptase and chain elongation of the viral DNA, resulting in inhibition of viral replication (235, 237). ZDV therapy in patients with advanced HIV-1 disease prolongs survival, decreases the frequency and severity of opportunistic infections, improves neurologic function, transiently improves CD4+ T-lymphocyte counts, and decreases the serum concentration of HIV-1 antigen (236,193,238-242). Anemia and neutropenia are the most frequent adverse reactions associated with ZDV therapy (243). In a double-blind placebo-controlled study of adults with AIDS and advanced AIDS-related complex, patients treated with ZDV had improved survival and decreased incidence of opportunistic infections (236). Further studies of this kind, in subjects with asymptomatic (245) and mildly symptomatic

HIV-1 disease and less than 500 CD4+ T lymphocytes/mm^3 also showed that ZDV therapy delayed progression of HIV-1 disease and produced little toxicity (244).

ZDV has shown beneficial effects in children and the Food and Drug Administration approved it in May of 1990 for pediatric use. Its pharmacokinetics (202) and adverse effects (172,173) in children are very similar to those in adults. In a multicentre phase II study from the AIDS Clinical Trials Group involving 88 children, commonly noted adverse effects were neutropenia (48%) and anemia (23%). In some cases, these complications reversed spontaneously but most required a dose reduction or discontinuation of the drug. Usually the dose is reduces to a half dose when the absolute neutrophil count is lower than 750 cells/mm^3 and discontinued when lower than 500 cells/mm^3 until the marrow function recovers. Some patients required blood transfusion.

Reported beneficial effects of ZDV in children have been weight gain, decreases in size of the liver and spleen, lowering of the total IgG and IgM toward more normal values, decreases in the amount of circulating p24 antigen in serum and cerebrospinal fluid, transient elevation in the numbers of CD4+ T lymphocytes (average 12 weeks), decreased frequency of virus isolation, and clinical (cognitive function) and radiologic improvement (brain growth) of HIV-1-associated encephalopathy (172,173,175,17,203). HIV-1-associated thrombocytopenia may improve at least temporarily with ZDV therapy, as reported in several studies in adults (192-194,17). There are no large studies in children but anecdotal evidence supports the same conclusions. Failure of ZDV to improve platelet counts in HIV-1-infected children has been reported (196).

A phase 1 evaluation of the safety, tolerability, and pharmacokinetics of ZDV administered to infants exposed at birth to HIV-1 was conducted. A total of 32 symptom-free infants were enrolled before 3 months of age. Infants under 2 weeks of age were administered oral doses of 2mg/kg every 6 hours and older infants were given 3mg/kg every 6 hours. It was found that ZDV at those doses in that age group was well tolerated, although some anemia (62.5%) and neutropenia (28.1%) were present, which generally resolved spontaneously (246).

ZDV at a dose of 180mg/m^2/dose every 6 hours is the recommended regimen for symptomatic children. Complete blood cell counts should be monitored closely throughout therapy and the dose adjusted accordingly.

Didanosine

Didanosine (ddI, Videx) (204-206) is an alternative nucleoside analog, which can be administered to children who cannot take ZDV, either because of intolerance or disease progression while on ZDV. A phase I/II, dose-ranging study by Butler et al. (204) investigated the clinical virological and immunological responses of 43 children, 16 of whom had been previously treated with ZDV and 27 of whom had not. In this study, 30% of the children showed an increase in CD4+ T cell counts. Weight gain exceeded 10% in 30% of the children and a reduction in lymphadenopathy and hepatosplenomegaly in 63 and 83%, respectively. Improvement in the parameters investigated was seen in both previously ZDV-treated and ZDV-naïve patients. Other beneficial effects reported were decreases in p24 antigen levels and resolution of thrombocytopenia. Stabilization of neurologic disease has been noted as well, and the drug penetrates the cerebrospinal fluid (206).

Preliminary data indicate that the drug is well tolerated. Another advantage of the drug is its longer dosing interval, being given at 200mg/m^2/day in two divided doses. Side effects include pancreatitis and neutropenia in small numbers of patients. Retinal changes have been observed in some didanosine-treated children. The significance of this finding is still unknown.

Zalcitabine

Experience with zalcitabine (ddC) is limited. One of the original trials (AIDS Clinical Trials Group Study 138) studied the efficacy and safety of zalcitabine monotherapy in 170 children with symptomatic HIV-1 infection in whom therapy with ZDV had failed (247). The children were randomly put on one of two treatment regimens: 0.005mg ddC/kg every 8 hours (n=86) or 0.01mg ddC/kg every 8 hours (n=84). Up to 18% of the children had previously shown intolerance of ZDV, while the remainder had developed progressive disease while on ZDV therapy. The efficacy endpoints investigated included CD4+ T cell counts, p24 antigen levels, weight changes and survival. Baseline CD4+ T cell counts were low in this group of children, with a median count of about 50 cells/mm^3. The median baseline CD4+ T cell count was 61 cells/mm^3 for the high-dose group and 44 cells/mm^3 for the low-dose group. CD4+ T cell count in both groups initially decreased from baseline until around 12 weeks in the low-dose group and until around 24 weeks in the high-dose group, then began to increase again toward baseline. By 36 weeks, the count had returned towards baseline values in the group taking 0.005 mg/kg, but not in the group taking 0.01

mg/kg. A 50% decrease in p24 antigen levels occurred in 65% of children taking low-dose ddC and in 57% of those taking the high dose. Over the 36-week period of treatment, almost all of the children showed an improvement in growth rate. Peripheral neuropathy did occur, but it was less common in those children than in adults receiving ddC, with only eight children out of 170 developing this condition. These overall findings indicate that ddC is generally well tolerated in this group.

Preliminary data on ddC indicate that neutropenia and pancreatitis with associated abnormalities in calcium and phosphate metabolism are the dose-limiting toxicities. Still, clinical and laboratory measures have improved in some children during therapy with ddC.

Combination Therapy

A study by Pizzo et al. (248) investigated the combination of ddC and ZDV in children previously treated with ddC therapy for 8 weeks. 25 patients started out with the ddC monotherapy. 13 of them were then switched to an alternating schedule of one week of ddC followed by three weeks of ZDV for 12-18 months. Of the 13 patients, 11 gained weight and more than half exhibited an increase of more than 10% in CD4+ T cell counts and the CD4:CD8 ratio. Additionally, four patients suffering from encephalopathy all improved on the combination therapy.

Husson et al. (249) investigated the combination of varying doses of didanosine with ZDV in a phase I/II study. 68 children who had not been treated previously or had developed intolerance to ZDV were enrolled. Eight dose combinations were investigated in the previously untreated children. A reduced dose of ZDV was used in children who had previously shown hematological intolerance to this drug. With the combination therapy, the geometric mean titres of cultured virus showed a highly significant decrease from baseline after 12-24 weeks of therapy, both in plasma and in peripheral blood mononuclear cells. There was a trend towards an increased weight for age in many of the children; 49% gained more than 10% of their baseline weight within 24 weeks. Additionally, there was a statistically significant increase in the mean CD4+ T cell count from baseline to 24 weeks. This effect was predominantly seen in the ZDV-naïve patients.

A pharmacokinetic evaluation of the combination of ZDV and didanosine was performed as part of this study (250). The area under the plasma concentration-time curve (AUC) remained unchanged when the

drugs were administered in combination as compared to when the drugs were administered singly. However, there was a greater interpatient and intrapatient variability with didanosine than with ZDV. Recently, Englund et al. (251) have suggested that in symptomatic HIV-1-infected children, treatment with either didanosine alone or ZDV plus didanosine was more effective that treatment with ZDV alone. The efficacy of didanosine alone was similar to that of the combination therapy and was associated with less hematologic toxicity. Over the past several years, significant progress has been made in antiretroviral therapy, both in children and adults. The standard of care has become the use of three or more agents, including members of new drug classes, such as non-nucleoside reverse transcriptase inhibitors and protease inhibitors. These are reviewed in (338). The focus of our work, as summarized in this thesis, has been based on children treated with nucleoside analogues, as extensively reviewed in this section.

Early vs. Late Fetal Transmission

Vertical transmission of HIV-1 from an infected mother to her child now accounts for virtually all new cases of HIV-1 infection in infants and children in North America and Europe. HIV-1 can be transmitted before, during, or after delivery. Table 3 provides a laboratory-based definition of early versus late transmission (329).

Table 3: Proposed Laboratory-Based Definition of Early vs. Late HIV-1 Infection[a]

Early (in utero) HIV-1 infection	Positive HIV-1 culture or PCR within 48h of birth[b]
Late (intrapartum) HIV-1 infection	Negative HIV-1 culture, PCR, or p24 antigen within one week of life and Positive HIV-1 culture, PCR, or p24 antigen between age 7 and 90 days[c]

[a] Modified from Ref. 84.
[b] Positive cord blood sample must be confirmed with sample from peripheral blood obtained within 48 h of birth. Second confirmatory sample obtained outside neonatal period should be positive by HIV culture of PCR.
[c] Infant must not be breast feeding.

Data are limited, however, about the relative proportion and efficiency of transmission during the intrauterine, intrapartum, or postpartum periods. The clinical observation that 20-30% of infected infants appear to develop rapid, early onset of AIDS during the first few months of life (and have presumably been infected for a longer period of time) provided initial indirect evidence for intrauterine transmission. The development of easily performed, reliable tests for the detection of HIV-1 proteins and nucleic acids in placental tissue and aborted fetal organs has provided more direct evidence for transmission during gestation.

Apparently, intrauterine transmission can occur during each trimester of pregnancy. HIV-1 has been identified by culture or polymerase chain reaction (PCR) in fetal tissue obtained from therapeutic abortions as early as 10 weeks gestation (58-61,35,75,39). HIV-1 has also been detected in placental tissue from HIV-1-infected women as early as 8 weeks gestation by techniques including ultrastructural examination, virus culture, immunocytochemistry, and *in situ* hybridization (34,77-79). Although contamination with maternal blood could potentially confound studies of placental or fetal tissue obtained from abortuses, many investigators have demonstrated the ability of certain placenta-derived cells to support HIV-1 replication *in vitro* (33,80,81).

Several investigators have detected virus in fetal tissue from abortuses obtained during the second trimester (286). Proviral sequences have been detected by PCR in fetal organs from abortions performed between 16 and 24 weeks gestation (35,76,93). Although tissue from 33 fetuses between 16 and 24 weeks gestation were analyzed, potential maternal blood contamination could be excluded in only nine (35). HIV-1 proviral DNA sequences were detected in fetal thymus (6/8), spleen (8/9), and peripheral blood (5/9) sampled from these nine fetuses. Although all nine fetuses had evidence of HIV-1 proviral DNA in one or more sites, all fetal organ samples were negative for HIV-1 by both virus isolation and p24 antigen testing. Lyman et al. (82) used PCR to detect proviral DNA in fetal central nervous system tissue from second trimester abortions. Proviral sequences were detected in 8/23 (30%) fetal organs. Maury et al. (33) have reported that placental tissue from both first trimester and term placentas expresses CD4 and can be infected by HIV-1 *in vitro*.

Other studies have failed to detect either HIV-1 in fetuses or in most infected infants at birth, suggesting that mother to infant transmission may also occur at or near the time of delivery (36). It is now felt that the majority of transmission events occur late in pregnancy or at parturition (37,22). Twin studies that show a greater chance of the firstborn twin of an HIV-1-infected mother becoming infected compared with the second born twin suggest that transmission of HIV-1 infection may occur perinatally (37). An updated analysis of 92 twin pairs showed that 30 pairs were discordant for infection. In 23 of 30 discordant twin pairs, the firstborn twin was infected, whereas in seven twin pairs the second born was infected (p=0.004). Therefore the presenting twin had a 3-fold greater risk of infection than the second born twin. Although this relationship was not influenced by the mode of delivery, risk of transmission was higher overall in

twins delivered vaginally than those delivered operatively. No data were available, however, regarding duration of ruptured membranes, length of labor, or indication for cesarean delivery. These data suggest that the presenting twin would have a prolonged exposure to infected blood and cervical secretions in the genital tract during the later stages of pregnancy and delivery. If direct mucocutaneous exposure to virus during delivery or ascending infection during labor are significant factors in HIV-1 transmission, preventive strategies aimed at reducing virus inoculum by methods such as viricidal lavage of the birth canal or immediate surface decontamination of the newborn might be utilized.

Although an increased risk of vertical transmission of HIV-1 has also been associated with prematurity (55,62,97), vaginal delivery (37,22,63,64,97), maternal viral load levels (287), and advanced immunodeficiency in the mother in some studies (55,62,63,66), such relationships have not been found by other investigators (37,67-69). There have been conflicting results from studies of singleton births regarding the influence of mode of delivery on transmission rates. Several investigators have reported similar infection rates regardless of mode of delivery (67,69,83). The European Collaborative Study found that cesarean delivery tended to decrease the risk of perinatal infection but only for emergency and not elective cesarean deliveries (63). This trend in relative risk reduction with cesarean delivery has also been reported by others, although reported reductions in transmission are marginal (64).

HIV-1 can also be transmitted postnatally through breastfeeding. The role of breastfeeding in postnatal transmission is clearly established for women who become infected after delivery, but the role of breastfeeding in women infected before delivery is uncertain (62,63,68,69,72). Although both free virus and proviral DNA have been found in breast milk (86,87), demonstration of breast milk transmission has been epidemiologically complex. Several studies have shown an increased risk of postpartum transmission by breastfeeding among women with primary HIV-1 infection during the peripartum period (67,87,72,88,89,90, 62). The infant, therefore, could be potentially exposed to secretions or cells containing a high viral inoculum.

Both high (67,62) and low (69) rates of HIV-1 transmission in breastfed infants have been reported. For example, the European Collaborative Study group reported a 2-fold increase in the risk of infection among breastfed infants (31 vs. 14%), but only 38 of 828 children evaluated were breastfed (63). A meta-analysis of published prospective studies estimated the additional attributable risk of transmission posed by

breastfeeding from mothers with established HIV-1 infection before pregnancy was 16% (95% confidence interval 8-25%) and was 26% (95% confidence interval 14-39%) from mothers who develop primary HIV-1 infection postpartum (63). Because of the risk of transmission and the availability of safe alternatives for infant nutrition, breastfeeding is not recommended in Canada for HIV-1-infected mothers.

Factors Associated With Risk of Transmission

Maternal factors influencing the rate of HIV-1 transmission from mother to child are incompletely defined. Indirect measures of increased viral burden, CD4+ T cell count, maternal disease stage, and absence of maternal antibody to specific HIV-1 epitopes have been associated with an increased risk of transmission. Additionally, characteristics of the maternal virus strains may also influence transmissibility. Advanced disease stage, either during pregnancy or within months of giving birth, has not often been associated with increased rates of vertical transmission in many studies (62,63,91,92). The presence of HIV-1-related symptoms combined with decreasing CD4+ T cell number was also associated with increased vertical transmission (94,62,108). A trend toward higher transmission for women with AIDS (31%) vs. asymptomatic women (14%) was found for the 615 women evaluated by the European Collaborative Study (94). A significant risk for transmission was found if the women were stratified by CD4+ T cell number (400/μl (19%), <700/μl (22%), and >700/μl (6%)) or CD4/CD8 ratios (>0.6 (12%) and <0.6 (24%)). Several studies have noted a correlation between p24 antigenemia and risk of vertical transmission (75,62). High p24 antigen coupled with low CD4+ T cell number or advanced disease stage has been linked with elevated transmission risk in several studies (95).

Several investigators have attempted to define the biologic and genetic features of the virus associated with perinatal transmission. In one study, the biological characteristic of the virus isolated from three transmitting and four non-transmitting mothers and their infants were evaluated (96). The virus isolated from the non-transmitting mothers grew more slowly and to lower levels than the isolates taken from mothers who transmitted the virus to their infants.

The HIV-1 genome is characterized by a high degree of genetic variability. The complex mixture of variants which exist in an infected individual are the result of competition and selection in response to

immunologic pressure for change, and to alterations in cell tropism and replication efficiency among the variants (98).

In an initial study to investigate the role of selection in perinatal HIV-1 transmission, the distribution of distinguishable genotypes transmitted between mother and child were analyzed (53). Comparisons of virus sequences from the three transmission pairs showed that specific sequences were highly conserved between each mother and her infant and that the infant's prevalent virus sequence was derived from a single variant present in its mother. Furthermore, the infants' virus sequences were less diverse than those of their mothers. Sequence sets from additional mothers and their infants have confirmed the observation that the infant's virus sequences are less diverse (96). This relatively narrow distribution is compatible with random transmission of a limited number of virions during gestation. Because of genetic evolution, these variants may be a minor form in the mother postpartum but represent a prevalent form found during gestation. Alternatively, there may be selection of an antigenically distinct variant in the mother that escapes a critical immune surveillance mechanism. Specific biological characteristics of the transmitted virus such as differences in cell tropism or replicative capacity may also be important.

HIV-1-specific humoral and cellular immune responses, while ultimately unsuccessful, inhibit virus replication and spread after infection and may be important in determining long-term disease outcome (99). Maternal HIV-1-specific immune response may be involved in preventing, or possibly enhancing, vertical transmission. Several investigators have proposed the level and specificity of maternal anti-HIV-1 specific antibody may be important in determining transmission. Antibodies to the hypervariable domain of the gp120 (the V3 region) have HIV-1 neutralizing activity in vitro. Mutations in this region have been associated with changes in cell tropism and neutralization escape (98). Several early studies have suggested that HIV-1-infected pregnant women with high antibody titres to conserved portions of the V3 hypervariable loop and/or high avidity-high affinity antibody against the principal neutralizing domain of the V3 loop may have a lower rate of HIV-1 transmission to their infants (55,56,66). However, these investigators evaluated antibody to V3 peptides that encompassed different areas of the V3 loop and more recent studies could not replicate these associations (71,100). Other factors, such as the maternal cytotoxic immune response to HIV-1, may be important in reducing transmission.

Additionally, enhancement of the maternal humoral and/or cellular immune response to HIV-1 through passive or active immunization, or both, may also be helpful. If protective virus epitopes that induce antibody with broad neutralizing capacity can be identified, passive immunization of the mother and/or infant with one or more monoclonal antibodies or active immunization with a subunit HIV-1 vaccine may provide optimal preventive interventions. If, however, there is selective vertical transmission of a maternal virus neutralization-escape variant, a polyvalent hyperimmune HIV-1 globulin preparation or vaccine will be necessary.

Fetal cell susceptibility to HIV-1 infection could vary by gestational age (possibly because of developmental differences in CD4 expression), and different fetal organ systems could vary in susceptibility to infection. Immature thymic cells have been shown to be readily infected with HIV-1 (101), and neonatal and cord blood macrophages have been found to be more susceptible to infection by HIV-1 isolates than adult macrophages.

If certain fetal cells or organ systems were particularly susceptible to HIV-1 infection, virus could infect these tissues (i.e. the thymus or central nervous system), escaping detection in peripheral blood samples obtained during the neonatal period. Infection of fetal stem cells may be more immunologically devastating than infection of more mature cells because of resulting stem cell destruction or dysfunctional cellular maturation, perhaps resulting in the more rapid disease course observed in perinatal HIV-1 infection when compared with HIV-1 infection in adults.

The potential role of the maturing immunologic capabilities of the fetus and fetal response to HIV-1 infection has not yet been evaluated; however, an immature immune system may be less able to restrict HIV-1 replication. A relative deficiency of circulating HIV-1 *gag*-specific cytotoxic T lymphocytes has been described in infants acquiring HIV-1 infection during gestation when compared with HIV-1-infected adults (102). It has been theorized that HIV-1 infection of early precursor thymic cells could lead to immunologic tolerance, inhibiting the ability to mount an effective immune response owing to perception of HIV-1 antigen as "self" (103).

Prevention and/or treatment of fetal infection may prove difficult. Indirect therapy to the fetus could be provided by transplacental passage of maternal antiretroviral therapy. However, toxicity to the developing placenta and fetus is a concern. Passive immunization of the fetus with neutralizing antibody could be

accomplished through transplacental active transport of antibody exogenously administered to the mother after the second trimester of pregnancy, or induced in the mother by active immunization. This approach should be prioritized in research settings.

Intensive exposure of the thin skin or mucosal surfaces of the fetus or newborn to maternal secretions during birth or through swallowing of infected amniotic fluid could provide a significant dose of virus. Modification of obstetrical practices could influence virus transmission occurring during the intrapartum period. If intrapartum transmission of HIV-1 occurs primarily through direct exposure of the infant to cell-associated or free virus in genital secretions, cesarean section performed before labor might be expected to reduce the risk of transmission. Additionally, virucidal cleansing of the birth canal before vaginal delivery and immediate surface decontamination of the infant by washing may provide more practical strategies to reduce transmission. However, if maternal-fetal blood exchange at the time of delivery is a significant source of virus, such measures might prove less beneficial. In the absence of prospective, controlled evaluations of operative delivery, current obstetric guidelines do not recommend cesarean section in HIV-1-infected pregnant women other than for standard obstetric indications.

Provision of antiviral therapy to the infant before intense virus exposure during delivery could reduce the risk of infection. Transplacental passage of antiviral drugs provided to the mother during labor could provide systemic antiviral activity in the infant at the time of exposure during delivery. Maintenance of antiviral activity in the infant for a period after delivery through short-term administration of an antiretroviral agent might be desirable. Connor and the Pediatric AIDS Clinical Trials Group Protocol 076 Study Group published a study in 1994 which indicated that in pregnant women with mildly symptomatic HIV-1 disease and no prior treatment with antiretroviral therapy during their pregnancy, a regimen consisting of ZDV given ante partum and intra partum to the mother and to the newborn for six weeks reduced the risk of maternal-infant HIV-1 transmission by approximately 67% (290).

The role of the newborn immune response to HIV-1 in averting transmission is unclear. Although one report noted reduced cellular immunity in HIV-1-infected infants (102), other researchers have noted relatively normal cell-mediated and humoral immune responses in HIV-1-infected infants during the first 2 years of life, with subsequent weakening (104). The presence of HIV-1-specific antibodies mediating

cellular cytotoxicity, neutralization and syncytium inhibition has correlated with slower disease progression in infected infants in several reports but obviously did not prevent transmission (105,106). Several factors potentially affect the transmission of HIV-1 from mother to fetus. These include 1) the placental barrier against HIV-1 transmission, 2) transfer of HIV-1-infected maternal leukocytes during pregnancy or during labor, in part influenced by placental pathology associated with AIDS, 3) qualitative and quantitative characteristics of the maternal virus, and 4) the nature of the maternal immune response against the virus.

Additional virologic characteristics may influence *in utero* transmission of HIV-1. It was noted that minor genetically distinct subsets of HIV-1 were selectively transmitted from infected mothers to their fetuses (53). Such observations imply the existence of either selective transmission of specific HIV-1 strains which can cross the placenta or HIV-1 strains with enhanced ability to infect the fetus. The phenomenon of selective cellular tropism of HIV-1 is well established. Thus it is conceivable that certain strains may have particular tropism for the placenta, and are subsequently passed on to the fetus.

The influence of specific anti-HIV-1 immune responses in determining the rate of transmission of the virus to the fetus is not clearly understood. Maternal gp120 antibodies have been reported to reduce transmission in several reports (37,66) but not in others (71). Low maternal anti-gp120 antibody titres have been associated with an increased risk for vertical transmission of HIV-1 (55). Initial studies (56) suggested a role for antibodies to the principal neutralizing epitope (V3) of HIV-1 in the transplacental transmission of the virus, but subsequent work shows no correlation (57).

Diagnosis of HIV-1 Infection in Children

Early diagnosis of HIV-1 infection in infants is essential to identify those patients who might benefit from early antiviral therapy, prophylactic treatment for opportunistic infections, along with aggressive treatment of bacterial infections, growth and development disorders, and psychosocial problems. Differentiating infected from uninfected infants will eliminate unnecessary procedures and therapy in those who are not, in fact, infected with HIV-1. Some early diagnostic tests may also provide information which helps establish the timing of transmission from mother to infant.

It is often difficult to diagnose HIV-1 infection in infancy, requiring a positive culture or direct viral detection assay results. These tests may be falsely negative during the first few weeks of life in approximately half of those infants who are subsequently shown to be infected. The presence of AIDS-defining symptoms and a declining CD4+ T cell count provides strong evidence for infection during infancy but it would be best to make the diagnosis at an earlier point in time to optimize the results of medical intervention. During the first 12-18 months of life, serologic testing of a child born to an HIV-1-infected mother will not be useful in determining the infection status of that infant because of the presence of maternal antibodies in the infant's circulation, acquired passively from the mother *in utero*. In infants younger than 18 months, tests for IgG antibody to HIV-1 in the circulation do not differentiate between infant and maternal antibody. This is quite important, as only 13-35% of infants will actually be infected. A more detailed discussion of diagnostic modalities and maternal/fetal HIV-1 transmission follows.

Antibodies to HIV-1

Serologic tests for HIV-1-specific antibodies are the mainstay of the laboratory diagnosis of HIV-1 infection in adults, children suspected of acquiring HIV-1 through nonperinatal routes (i.e., blood or blood products), and children with suspected perinatally acquired infection who are older than 18 months. IgG antibodies are generally detectable within 4-12 weeks after exposure (111) and throughout the entire course of the disease; however, patterns of antibody production to certain viral proteins may change over time.

The most common antibody screening tests are enzyme-linked immunosorbent assays (ELISA) used in conjunction with confirmatory tests such as the Western blot or immunofluorescence assay (IFA) to confirm the specificity of ELISA reactions.

Studies have shown that virtually 100% of infants born to seropositive mothers will test antibody positive at birth, but only 20-30% will be infected. Those who are uninfected lose maternal antibody usually between 6 and 12 months of age, but a small proportion may retain maternal antibody for up to 18 months (63,67,112,279).

A positive antibody test alone identifies perinatally-exposed infants. Careful follow-up and management is warranted for those cases. Clinical evaluation with repeated testing over at least the first 2 years of life has been the primary means of establishing the diagnosis in these infants. Infants who become antibody

negative (seroreverted) and remain well are generally considered uninfected, although there have been a few reports of asymptomatic infants who have seroreverted but have positive virus tests such as culture or PCR (113). In addition, rare reports have described perinatally infected children who had lost maternal antibody and later seroconverted (114,115).

Repeat Western blot testing over the first year of life and comparison of the band pattern over time can occasionally identify an infected infant. The infant's band pattern at birth is usually identical to the mother's pattern, since most of the detectable antibody is of maternal origin. The appearance of new bands on postnatal samples that were not present in the birth sample indicates de novo production of antibody by the infant (116).

Because maternal IgM and IgA antibodies do not cross the placenta, the presence of these HIV-1-specific antibodies can be used to indicate the presence of HIV-1 infection in infants. Several studies indicate that HIV-1-specific IgA assays can detect most HIV-1-infected infants by the age of 6 months but are generally negative in infected infants younger than 3 months, presumably because HIV-1-specific IgA antibody is not yet produced in sufficient quantity (52,117,280). Less success has been obtained using HIV-1-specific IgM assays, presumably because production of this antibody type in infants is more transient and at lower serum levels (65).

In Vitro Antibody Production Assays

Another potential diagnostic test is the *in vitro* antibody production assay (IVAP). This assay detects the presence of HIV-1-specific antibody-producing B lymphocytes in the infant, which indicates that the infant's immune system has been stimulated by HIV-1 infection. The standard IVAP assays require 7-10 days to complete.

Two methods for this technique have been described. In one method, PBMCs are separated from whole blood, carefully washed to remove plasma, placed in medium, and stimulated to produce antibody with either pokeweed mitogen (127) or Epstein-Barr virus (50). HIV-1-sensitized B cells will produce antibody that is released into the culture supernatant, and can be detected using standard methods.

There are some important limitations to this assay. False-positive tests in uninfected infants have been reported in the first 2 months of life. These spurious results might result from the detection of maternal B lymphocytes that are producing antibody but may not harbor the virus. Additionally, in the presence of

abundant maternal IgG in the infant's serum, false-positive tests may result from maternal anti-HIV-1 antibody that has adhered to infant B cells in the culture. This method is not currently used in clinical practice.

p24 Antigen Assay

The standard p24 antigen assay has been a helpful but limited method for diagnosing pediatric HIV-1 infection. Studies of infants born to HIV-1-infected mothers have found very few infants to be antigen positive early in the course of infection, because of the apparent low levels of antigen in the first month of life and the presence of excess maternal antibody which complexes to any free p24 antigen that is present (112,118). Despite these limitations, the antigen test can be helpful in certain settings. Hypogammaglobulinemic infants with HIV-1 infection can have positive antigen tests (119). Recent studies have shown that modification of the standard p24 antigen assay by acidification of the sample to dissociate the immune complexes can increase the sensitivity of the assay to detect HIV-1 infection in infants (122,123). The immune complex dissociation p24 antigen assay has been shown to be highly sensitive and specific for diagnosis of HIV-1 infection in infants, although samples taken in the first week of life have been somewhat problematic in that false-positive and false-negative tests have been observed.

HIV-1 Culture

Virus culture is one of the most sensitive techniques for detecting HIV-1 infection in infants and is used extensively in research and clinical settings. Micrococultures in 12-, 24-, and 96-well plates are equally sensitive and specific compared with standard flask methods and require far less blood (18). HIV-1 culture is not useful as a rapid diagnostic test, because cultures typically take 7-28 days or more to complete. The standard technique involves coculturing patients' PBMCs which have been isolated from whole blood with phytohemagglutinin-stimulated PBMC feeder cells from healthy uninfected donors. At least $2x10^6$ patient cells should be cocultivated with an equal number of donor cells. The cocultured cells should be stimulated with T cell growth factor (interleukin-2), which enhances viral growth. Cultures must be supplemented every 3-5 days with fresh feeder cells and monitored at least weekly for the presence of virus by measuring if HIV-1 is present in culture supernatants (124,125).

Techniques which specifically quantitate the amount of HIV-1 growing in culture have also been developed. The technique involves coculturing serial dilutions of patient PBMCs (separated from whole blood) with a constant number of uninfected donor cells (127). Cultures are then monitored in the same way as described above. The lowest dilution of cells required to produce a positive culture is the end point, and the titre of HIV-1 is expressed as the tissue culture infective dose per 10^6 cells. Plasma viral loads can also be measured using serial dilutions of plasma cultured in the same manner as above and results expressed as tissue culture infective dose per millilitre plasma (127). By these techniques, studies have found that the mean viral titres in plasma were lowest in asymptomatic HIV-1-infected patients and higher in symptomatic patients (127). Likewise, the percentage of infected PBMCs was also lowest among asymptomatic patients and higher among symptomatic patients. This assay is more commonly used for clinical monitoring than as a diagnostic test.

Polymerase Chain Reaction

PCR is the most sensitive diagnostic technique for detecting HIV-1. DNA sequences in PBMCs can readily be detected in infants. The test only requires ~ 1ml blood and can be completed in a matter of hours (283). Studies evaluating the use of PCR for early diagnosis of HIV-1 infection have shown that ~30-50% of HIV-1-infected infants will test positive close to the time of birth (112,22). This percentage increases to nearly 100% by age 1-3 months. These sensitivities are comparable with those of virus culture (125). PCR can also be used to measure active virus replication by detecting viral RNA in plasma (22). Several studies indicate that infants who lose maternal antibody and remain healthy are uninfected based on negative virus cultures and PCR (126). Some laboratories have reported rare seroreverting children who will occasionally test positive by PCR on a single specimen, but negative on subsequent specimens (126, 118,281,282). The reasons why these infants tested positive on one occasion are unclear. A mix-up in specimens or other laboratory errors is one possible explanation (282). Alternatively, transient PCR positivity may represent 1) true infection that has cleared, 2) persistent infection that is below the level detectable by the test, or 3) maternal blood cell contamination. Continued evaluation of these children is important.

Surrogate Tests-Immunologic Parameters

Tests which measure immunologic abnormalities most commonly associated with HIV-1 infection can also be useful for the diagnosis of infection in infants. According to the CDC classification system for HIV-1 disease in children, the combination of both humoral and cellular immunodeficiency in a symptomatic infant born to an HIV-1-infected mother is diagnostic of HIV-1 infection in the absence of specific diagnostic tests (224). The classic immunologic abnormalities include low T helper (CD4+) lymphocyte counts (adjusted for age), elevated T supressor (CD8+) counts (particularly early in life), reversed CD4:CD8 ratio; depressed lymphocyte responses to mitogens *in vitro*, strikingly elevated immunoglobulin levels (most commonly IgG) or (rarely) hypogammaglobulinemia, and decreased specific antibody responses. CD4+ T cell counts are used to monitor immunosuppression and disease progression over time. The most important factor which must be considered when choosing assays for the diagnosis of perinatal HIV-1 infection is the age of the infant at the time of testing. Although the precise kinetics of viral replication and antibody production in infants remain to be defined, several studies indicate that virus load is probably lowest at the time of birth in most infants and increases during the first few months of life (125,22,112). IgA antibody production appears to also be low during the first few months of life (117,52). IVAP assays may yield false-positive results in the first 1-2 months of life. Thus, in the neonatal period, most studies have shown that the greatest percentage of infected infants are detected using sensitive virologic assays such as PCR or virus culture (284). Several assays can detect infection by age 3-6 months (128). Even the most sensitive assays can detect no more than 50% of infected infants around the time of birth. This is likely because the viral load is extremely low, virus is suppressed or sequestered in other tissues, or infection has only recently been transmitted during labor. Table 4. summarizes the relative sensitivity of early diagnostic tests for HIV-1 infection in infants from birth to 6 months (335).

Table 4: Sensitivity of Early Diagnostic Tests for HIV-1 Infection in Infants

	Time to Detection				
Laboratory Method[a]	**1 week**	**2-4 weeks**	**1-2 months**	**3-6 months**	**>6 months**
HIV-1 IgA antibody testing (ELISA)	<10%	10-30%	20-50%	50-80%	70-90%
ELISPOT				>95%	>95%
IVAP				>95%	>95%
HIV-1 p24 antigen	10-25%	20-50%	30-60%	30-50%	20-40%
PCR	30-50%	50%	70-90%	>95%	>95%
Viral culture	30-50%	50%	70-90%	>95%	>95%

[a]HIV-1 IgA antibody testing (ELISA/Western blot) not useful for detection of HIV-1 infection.

The stage of disease also affects the likelihood that a given assay will detect the presence of HIV-1 infection. As disease progresses, the virus load both in the plasma and cells increases (127,19). Some severely immunosuppressed adults and children with end-stage disease may lose antibody but this is highly unlikely to occur in very young infants. Some perinatally infected infants may be hypogammaglobulinemic and will not produce antibody and this should be considered in appropriate settings.

However, the timing of transmission may also affect the likelihood of early detection of infection in perinatally infected infants. Theoretically, infants infected *in utero* should be positive at birth, whereas those infected late in pregnancy and during the intrapartum or postpartum periods may not become positive until sometime after birth. One study evaluating PCR, virus culture and plasma p24 antigen tests for diagnosis of HIV-1 infection in children under the age of 6 months found that PCR and culture were comparable in sensitivity, detecting 90% of all positive specimens. Both assays found HIV-1 in only half of infected newborns, suggesting that this fraction of children was infected during gestation, and the rest were infected at or near the time of delivery. Plasma p24 antigen was detected in 75% of all samples tested but in only half of infected children during the first 2 months of life and 88% of samples from children during the next 4 months (285).

With respect to immunologic assays, low CD4 count and high Ig (particularly IgG) levels in a child with physical signs of HIV-1 infection (diffuse lymphadenopathy, hepatosplenomegaly) and a possible source of exposure to HIV-1 are very suggestive of HIV-1 infection. β_2-microglobulin and neopterin levels have also been suggested as potentially valuable markers for diagnosis of HIV-1 infection in children (288,289) but they are too non-specific for widespread use.

Prenatal diagnostic techniques available today, such as ultrasound, amniocentesis, chorionic villus sampling (CVS), cordocentesis, fetal blood sampling, and embryoscopy may allow physicians to identify fetal HIV-1 infection early in gestation. This would allow HIV-1 seropositive women to better decide whether to carry or terminate their pregnancies. Furthermore, as therapeutic agents become available for use in pregnancy, the HIV-1 status of the fetus may be used in deciding if and when to start therapy and in assessing responses to therapy. There are, however, several important questions which will need to be

answered before prenatal HIV-1 diagnosis will be conducted routinely. It must first be determined that an HIV-1-infected fetus can be detected with a high degree of accuracy. The risk of both missing the diagnosis in an infected fetus and making the diagnosis of fetal infection because of maternal contamination of fetal specimens must be greatly reduced. Finally, it is crucial that the techniques used have virtually no risk of infecting an otherwise healthy fetus. Although the technology may exist to satisfactorily achieve these goals, the tests we have today do not provide any safe or reliable prenatal diagnostic methods for HIV-1 infection of the fetus.

Virologic Hypothesis of HIV-1

Initial infection in older children and adults can be asymptomatic or result in an acute, self-limiting mononucleosis-like syndrome occurring 2-4 weeks after infection and resolving within 1-2 weeks (11,12). Both the acute HIV-1 syndrome and asymptomatic infection are followed by production of anti-HIV-1 antibodies, termed seroconversion, which usually appear 6-12 weeks after infection. High levels of infectious HIV-1 have been demonstrated in both the plasma and peripheral blood mononuclear cells (PBMC) (15, 16) of infected individuals during primary infection, suggesting that the initial viral burden may be rather high prior to the development of an immune response to the virus. In fact, such a response does develop, leading to the restriction of viral replication and the establishment of chronic infection (16). In general terms, lower levels of HIV-1 are found in the plasma and PBMCs of asymptomatic or mildly symptomatic children whereas severely symptomatic children may have a virus burden comparable to that found in adults (17-19). However, in studies by Saag et al. (20), viral levels in the plasma of each of five children infected in the perinatal period did not appear to relate to immune disease or duration of infection. This is in marked contrast to findings in adult patients, where levels of plasma viremia decrease rapidly after seroconversion and remain relatively low throughout the asymptomatic phase of infection (324). Thus, significant differences in the virology of HIV-1 may be present in infected adults, as compared to children.

In addition, a shift has occurred in the accepted thinking about how HIV-1 coexists with its human host. Previously, it was thought that following seroconversion, a period of latency was entered, during which time little viral replication occurred. Fig 3 shows a generalized picture of the pattern of HIV-1 infection *in*

vivo according to this model. Following primary infection, an extended period exists which lasts 2-15 or more years and is characterized by low but steady viral loads in the circulation (252). This time seems to be a period of relatively little activity, however, recent data (summarized below) have shown this to be a time of extensive viral replication and cell killing. Recognition of this phenomenon has allowed a major shift in our understanding of the virus-host interaction. In HIV-1 infection, we now have to think of AIDS as being the end product of damage accumulated during the entire course of infection, even while the patient seems healthy, rather than a "threshold" phenomenon developing in isolation as the host's immune response to HIV-1 suddenly collapses.

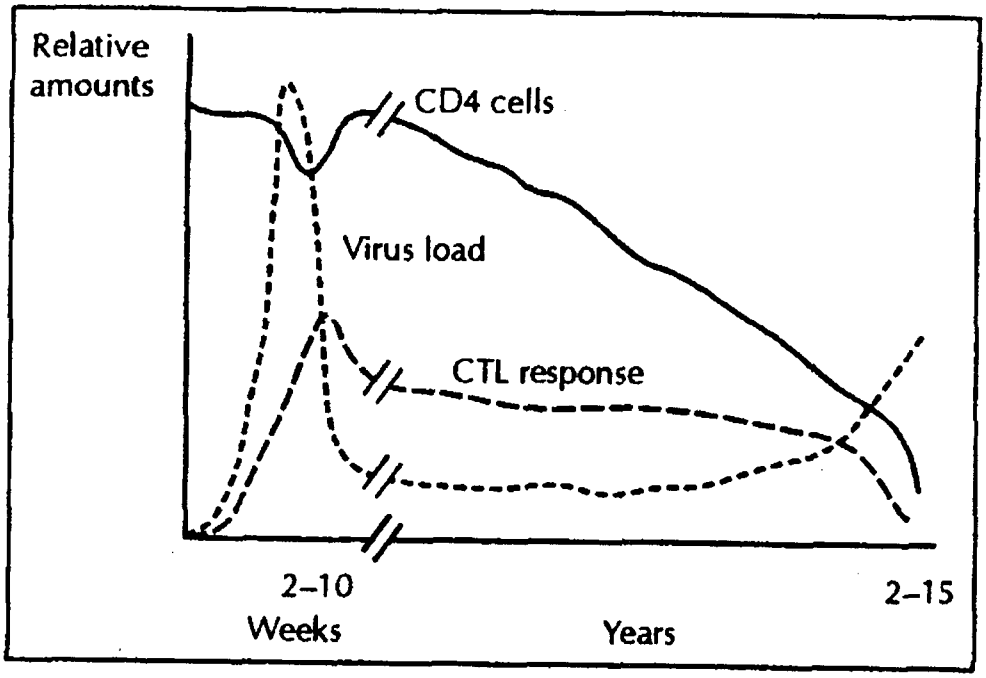


Fig. 3: The pattern of HIV infection *in vivo*. Shown is the typical picture observed. There is considerable variation from patient to patient (252).

The fact that genetic diversity accumulates very rapidly in HIV-1 populations growing in an individual was the first clue that the clinically latent period might be a time of rapid ongoing virus replication (253). The only explanation for how so much diversity could accumulate is that viral turnover continues during the time of relative equilibrium. Why then isn't the virus load in an infected individual constantly increasing?

The steady state concept was conceived to explain this phenomenon (254). In this case, the rate of infection of new cells equals the rate of death of infected cells. The rate of cell replacement equals the rate of death of infected plus uninfected cells. The amount of circulating virus equals the balance between the rate of virus release into the blood and its rate of clearance from the blood, and the rate of virus release is proportional to the number of infected virus-producing cells (255). This new steady state concept is of paramount importance. In its simplest form, the steady state can be visualized as a collection of cells with similar kinetic rates of infection, virus production, and cell death. For every cell infected, one infected cell dies, each infected cell makes on average the same amount of virus, and for each virion released into the blood, one virion is removed from the blood. Real life is, of course, much more complicated that this. Examining numbers of infected cells or the amount of virus in the blood (256,257) as a snapshot, does not disclose the underlying dynamics. In order to understand the dynamics, the system has to be disturbed and the response to that disturbance measured. Introducing antiretroviral therapy is one way to disturb the system.

In studies performed on viral production in such systems, the number of virus-producing cells declines with kinetics which reflect their natural half-lives as well as the clearance rate of free virus. Several papers were published based on these studies (258-260), leading to the generation of mathematical models to summarize viral dynamics in the infected host. The half-life of the circulating virus population in the steady state is 1-1.5 days. The population of wild-type viruses is decreased to as little as 1% of its original level following ~ 1 week. If any viral replication is allowed to persist in the presence of drugs, almost all genomes present in the circulation after ~ 2 weeks contain mutants which are resistant to one or more of the drugs being used, and viral load approaches pre-treatment levels. Another group of more refined studies (261) has helped us expand on these conclusions. The overall average replication cycle is approximately 2 days. This means that 180 generations pass per year, and that 1000 generations pass in 5-6 years. Less than 1% of the virus in blood at any time comes from cells infected more than 2 weeks previously. This means that at most, only a minor contribution comes from latently or chronically infected cells. The kinetic parameters seem to be independent of the clinical state. In addition, the steady state is quite vigorous over a wide range of virus replication efficiencies.

There are controls in place which maintain the steady state. The balance between immune response and virus replication, between immune response and antigenic variation (262), and the availability of target cells (255,263) is somehow maintained. Understanding and modeling the balancing forces which keep HIV-1 infection under control for such a long time in spite of the rapid dynamics of infection is a research aim of major importance.

Recently, the significance of plasma viral load in relation to this new model of viral dynamics has been elucidated. It has been clearly shown that there is an excellent correlation between the steady state level of virus in blood and the probability of disease progression. Mellors et al published an elegant study which showed that higher viral loads present at the time a patient is diagnosed predict much more rapid disease progression (264). They also showed that plasma viral load was a strong predictor of rapid progression independent of CD4 T cell count (265). These data have been confirmed in patients receiving antiretroviral therapy, with a 50% reduction in HIV-1 RNA level being associated with a 27% decrease in the adjusted risk of disease progression (266). Additional evidence to support this has been presented by Jurriaans et al. (273), Henrard et al. (274), and Katzenstein (325).

It also appears that the level of plasma HIV-1 viral load also predicts the progression rate of disease in children (267-272). Shearer et al. (276) also showed that the level of plasma HIV-1 viral load in infants predicted risk for disease progression and have demonstrated that in perinatally infected infants, HIV-1 RNA levels are high and decline more slowly than adults during the first two years of life. Infants with very high viral loads are at increased risk for a rapid progression of disease, which suggests that early treatment with antiretroviral agents may be indicated in the first few months of live to reduce this risk.

Viral dynamics as they are now understood have significant implications in terms of current therapeutic strategies. Until recently, all antiviral therapy showed a similar response in viral load, which was not sustained, due to the potency of the agents and regimens being used. More recently, therapeutic regimens have been described which give rise to greater viral load suppression (277,278). If a treatment can be found which suppresses viral replication even more efficiently (perhaps completely), and sustains that suppression, it may lead to a long-term remission and the delay in immune disease progression, perhaps indefinitely. Although such a treatment does not yet exist, we all live in anticipation of its development.

Antiretroviral Drug Resistance

An important feature of retroviral reverse transcriptases is that they do not have the ability to edit errors in transcription which occur during nucleic acid replication. Based on newly understood HIV-1 viral dynamics, the high rate of viral turnover in HIV-1 infection indicates that each single-point mutation may arise 10^4 to 10^5 times each day (301). The selective pressure exerted by antiviral therapy promotes the emergence of strains of virus which are resistant to the drug being used. Such resistant isolates have been found in clinical samples from patients receiving all of the currently approved nucleoside analogue reverse transcriptase inhibitors. For several of these agents, the emergence of drug-resistant strains has been associated with loss of antiretroviral activity and disease progression (302).

There are several factors, including particular properties of the virus, host, and drug, which combined determine the rapidity with which drug resistance develops, and thus the duration of clinical benefit for each individual. Faster development of resistance is associated with low CD4+ T cell counts, more advanced HIV-1 disease and high plasma viral loads. The influence of the syncytium-inducing phenotype is currently the subject of some debate (303). The development of resistance is also dependent on the combination of drugs which the patient is receiving. The pressure on the virus to develop resistance is proportional to the potency of the drug combination in the patient. Combination therapy is extremely important, because none of the currently available drugs has the ability to completely suppress viral load when administered as a monotherapy. Thus, the goal of current therapies is to find the correct combination of drugs which have the ability to achieve suppression of virus replication (viral load) to a level which delays the emergence of resistance.

Zidovudine Resistance

ZDV was the first nucleoside analog to be introduced to treat patients with HIV-1 infection. As previously noted, it was shown to prolong survival, decrease the frequency and severity of opportunistic infections, improve CD4+ T lymphocyte counts and decrease serum HIV-1 p24 antigen concentration and the titre of infectious HIV-1 in plasma in patients with advanced HIV-1 infection (236,193,127). It was also shown to delay disease progression in patients with mildly symptomatic HIV-1 infection and asymptomatic HIV-1-infected individuals with peripheral blood CD4+ T lymphocyte counts $<500x10^6$ cell/μl (244,245,312). However, most of these beneficial effects were of limited duration (236,193,311). Within two years of the

introduction of ZDV, strains of HIV-1 with 100-fold decreased susceptibility to this agent were isolated from patients who had been on prolonged therapy (215,313,314). In these patients, the median drug concentration required to reduce viral replication by 50% (IC_{50}) increased from an average of 0.003μmol/l before therapy to 3.0μmol/l after 18 months of treatment (298) (phenotypic resistance defined as IC_{50}>1μmol/l). ZDV-resistant HIV-1 can be detected as early as 6 months after the beginning of therapy and about 50% of patients have resistant isolates after 2 years (298).

There were originally four mutations identified in the HIV-1 reverse transcriptase sequence which were associated with ZDV resistance. These were: D67N, K70R, T215Y/F, and K219Q (215). A fifth mutation associated with ZDV resistance, M41L, was subsequently described (304). The order in which these mutations appeared was also subsequently elucidated. In the typical patient, there is a transient appearance of the K70R mutation; its disappearance is paralleled by the appearance of the T215Y/F mutation. After prolonged therapy the K70R mutation may reappear in parallel to the D67N mutation (305). Over time, high-grade phenotypic resistance develops with all five mutations being present if ZDV therapy is continued. The most reliable and durable marker of disease progression is the presence of the mutation at codon 215.

The presence of ZDV resistant mutations in patient isolates are associated with CD4+ T cell decline and clinical failure on ZDV monotherapy, and decreased clinical responses to subsequent therapy with other nucleoside-containing drugs or drug combinations (306,307). In the AIDS Clinical Trials Group (ACTG) 116B/117 trial, high level phenotypic resistance to ZDV at baseline was an independent prediction of disease progression or death, with a relative hazard ratio of 1.93 (213). The use of ZDV is now so widespread that transmission of already ZDV resistant virus has occurred by sexual, percutaneous (in a healthcare worker) and maternal-infant routes (308-310). Transmission of such an isolate from one child to another has also been documented (319).

Few studies have been published on ZDV resistance in children. In 1993, a study of 19 children showed that individuals with ZDV resistant strains had worse clinical outcomes than children whose viruses remained susceptible, as determined by a 50% decline in absolute CD4+ T cell counts after one year of treatment, failure to thrive, or death. There is an urgent need to confirm these findings in larger, more varied pediatric patient populations.

Resistance Testing Methodologies

Antiretroviral drug susceptibility assays are divided into two groups, genotypic and phenotypic. The first involves isolating RNA or DNA from biological materials to determine the presence or absence of specific mutations associated with drug resistance (215-218). The second involves growing HIV-1 *in vitro* and measuring its replication characteristics in the presence of a given drug. In a prototypic method, HIV-1-infected peripheral blood mononuclear cells (PBMCs), with seronegative phytohemagglutinin-stimulated donor PBMCs to obtain an HIV-1 stock are incubated. The virus stock is then titrated for viral infectivity (50% tissue culture infective dose) by use of serial fourfold virus dilutions in donor PBMCs. A standardized inoculum of 4,000 50% tissue culture infective doses per 10^6 cells is used in a second step of the procedure to acutely infect seronegative donor PBMCs in a 7-day microtitre plate assay with triplicate wells containing ZDV concentrations ranging from 0 to 5.0 μM. The 50% inhibitory concentration (IC_{50} of ZDV) is then determined (212-214,124,127). An isolate is deemed sensitive if the IC_{50} is < 0.2 μM and deemed highly resistant if the IC_{50} is >1.0 μM.

A third method is a variation of a quantitative micrococulture outlined in the ACTG Virology Manual for HIV-1 Laboratories (124). The assay is less expensive and time consuming than the standard phenotypic resistance assay. The infected PBMCs from patients are isolated and directly cultured with PHA-stimulated negative donor PBMCs (322). Six 5-fold serial dilutions of PBMCs were prepared in duplicate and to one duplicate was added ZDV to a final concentration of 5 μM. The cultures are maintained for 14 days, with new drug and PBMCs added on day 7. P24 antigen levels in the supernatants are then determined. A reduction in viral replication in the row of wells containing ZDV compared to the parallel row of drug-free control wells was taken as a crude measure of ZDV susceptibility. This assay can be adapted to test susceptibility to other antiretrovirals, allowing for the direct evaluation of drug susceptibility over 14 days, minimizing the selective effects of *in vitro* passage that may be problematic in the macro-culture methodology.

The use of both genotypic and phenotypic drug resistance testing may provide us with complimentary information and it would be useful to evaluate both methods in parallel to establish the usefulness of the information they generate.

Research Hypothesis

Many clinical and laboratory measures exist which produce information to allow physicians and care givers to have a better understanding of an HIV-1-infected child's disease status, and their risk of progressing to AIDS. These measures include viral load testing, drug resistance testing, monitoring of CD4+ T cell levels, viral phenotype testing (as some phenotypes grow more rapidly and aggressively than others (296, 297, 303, 321, 325)), along with regular evaluation for clinical disease progression. There are many practical reasons why it is impossible to perform every test on every patient at every point in time. Therefore, it is imperative, to make the best use of time and resources, that the most accurate and informative tests (singly or in combination) be found to monitor the disease state of children infected with HIV-1. In this study we hypothesized that an algorithm comprising a group of clinical and/or laboratory tests exist which would be the best markers by which to assess a pediatric HIV-1 patient's current disease status as well as the risk of disease progression over time.

Specific Objectives

1. To determine the prevalence of resistance to ZDV in a cross-section of HIV-1-infected Canadian children and to compare different resistance testing methodologies
2. To determine the association of drug resistance with clinical and laboratory variables including:
    a. Cell-associated viral load
    b. Plasma viral load
    c. Viral phenotype
    d. CD4+ T cell count and CD4+ T cell percent
    e. Presence or absence of HIV-1-related symptoms
    f. Presence or absence of HIV-1-related encephalopathy
    g. Mode of acquisition of HIV-1

MATERIALS AND METHODS

Patient Population

A total of 103 patients were recruited from seven centres across Canada: Izaac Walter Killan Hospital in Halifax, St. Joseph's Health Centre in London, St. Justine's Hospital in Montreal, The Children's Hospital in Montreal, The Children's Hospital of Eastern Ontario (CHEO) in Ottawa, The Hospital for Sick Children in Toronto, and British Columbia Children's Hospital in Vancouver. All patients were know to be infected with HIV-1 and between the ages of 3 months and 18 years of age at the time of enrollment.

Specimen Collection

Approximately 10.0ml of anticoagulated whole blood was collected in heparinized tubes from each patient at their respective clinics. The samples were then packed and transported in saf-T-paks® by either air or ground transport according to the Canadian Transportation of Dangerous Goods Regulations (TDG Regulations) to our laboratory within 24 hours of the sample being drawn. The samples from CHEO were processed on the day the specimen was drawn, within six hours of sample collection.

Isolation of Lymphocytes

Isolation of lymphocytes was performed in a BSL-3 containment facility in a laminar flow hood (18,19,124). Upon receipt of a specimen, the tube was inverted eight times and centrifuged at 400 x g for 10 minutes. A total of 2.0-3.0ml plasma was removed and placed into 1.5ml screw cap tubes in 1.0ml aliquots and frozen at -70°C to be used later for plasma viremia measurements. The volume of removed plasma was then replaced with Phosphate Buffered Saline (PBS) at room temperature and the contents of the tube was mixed. The blood was then layered slowly onto Ficoll-Hypaque® solution (Pharmacia, Uppsala, Sweden or Piscataway, New Jersey, USA) (blood volume to Ficoll volume ratio 4:3) in a 15 or 50ml polystyrene centrifuge tube and centrifuged at 400 x g for 30 minutes at 22°C without a brake. The remaining supernatant was removed and discarded and the layer of peripheral blood mononuclear cells (PBMCs) was then carefully removed, then mixed with 40.0ml 1xPBS at room temperature and centrifuged at 400 x g for 15 minutes. The PBS was removed and the pellet of cells was then resuspended

in 10.0ml PBS, 50µl of the suspension was removed for the enumeration of cells (by Trypan Blue dye exclusion) and the remainder was centrifuged at 60-100 x g for ten minutes.

After the spin, the supernatant was discarded and the cells were resuspended in 1xPBS or R-3 medium [RPMI-1640 (GIBCO Laboratories, Grand Island, New York, USA) with 10% heat-inactivated fetal bovine serum; 2mM L-glutamine; 5% interleukin-2 (IL-2; Boehringer Mannheim, Brussels, Belgium); penicillin (100units/ml)/streptomycin (100µg/ml); 10mM HEPES Buffer] to a concentration of 1.0-3.0x10^6cells/ml, depending on the number of cells obtained. For storage, a minimum of 1.0x10^6 cells were saved in 1.5ml screw cap tubes (1.0-3.0x10^6cells/tube). The tubes were centrifuged, the supernatant removed, and the remaining pellets were stored at -70°C to be used at a later date for proviral load measurement or sequencing.

Qualitative Macrococulture Assay

From the PBMCs isolated, a separate aliquot of 6.0x10^6cells was removed after the final centrifugation above and transferred to a fresh 15ml polystyrene centrifuge tube. These patient cells were used to prepare a qualitative PBMC macrococulture assay, according to the AIDS Clinical Trials Group (ACTG) Virology Manual, September 1994 edition (124,207,208). The concentration of the cells was adjusted to 2.0x10^6cells/ml in R-3 medium. One to three day old, HIV-1-negative, PHA-stimulated donor PBMCs were sedimented in a 15ml polystyrene centrifuge tube at 400 x g for 10 minutes at 22°C. The cells were then resuspended in R-3 medium and enumerated by Trypan Blue dye exclusion. The cells were adjusted to a concentration of 2.0x10^6cells/ml. 6.0x10^6 donor PBMCs were required for each sample (3.0ml cells in R-3 medium at 2.0x10^6cells/ml). The donor cells were combined with the 6.0x10^6 patient cells (3.0ml cells in R-3 medium at 2.0x10^6cells/ml) in a 25cm^3 flask (total volume: 6.0ml). The flask was then incubated on its side in a humidified chamber at 5% CO_2 and 37°C for 21 days.

On days 3, 10, and 17, the flask was removed from the incubator, examined under the light microscope for fungal contamination and set upright in a laminar flow hood for 20 minutes. Approximately 3.0ml of supernatant was removed from the top of the culture and frozen in 1.0ml aliquots in 1.5ml screw cap tubes at -70°C. The volume of supernatant removed was replaced with fresh R-3 medium. The suspension was mixed and the flask was then returned to the incubator.

On days 7 and 14, the flasks were removed from the incubator, examined under the light microscope and set upright in a laminar flow hood for 20 minutes. Approximately 3.0ml of supernatant was removed from the top of the culture and again frozen in 1.0ml aliquots in 1.5ml screw cap tubes at -70°C. The volume of supernatant removed was replace with 1-3 day old, HIV-1-negative, PHA-stimulated donor PBMCs which had been centrifuged at 400 x g for 10 minutes at 22°C, enumerated, and resuspended in R-3 medium to $2x10^6$cells/ml. On day 21 the culture was terminated. Again, 3.0ml of supernatant was removed and frozen as above. All of the collected frozen supernatants were later thawed and HIV-1 p24 antigen levels were measured using commercial p24 assay kits (Organon Teknika BV, Boxtel, The Netherlands or Coulter, Hialeah, Florida, USA), as a measure of ongoing viral replication. From these stocks, viral phenotype assays and conventional HIV-1 drug susceptibility assays were performed, as described below.

Quantitative Microculture Assay

A final cellular aliquot (minimum of $2.7x10^6$ isolated patient PBMCs) was then used to set up a quantitative PBMC microculture assay (124). The isolated PBMCs from each sample were enumerated and diluted to a concentration of $1.0x10^6$cells/ml in a sterile 15ml conical centrifuge tube, which was labeled tube A. To each of five additional tubes, labeled B through F, was added 2.4ml R-3 medium. From tube A, 0.6ml of suspended patient PBMCs were removed, transferred to tube B, and mixed. A fresh pipette was then used to remove 0.6ml of cell suspension from tube B and to transfer it to tube C. After thorough mixing, the procedure was repeated for the next four dilutions. The final dilutions in tubes A through F contained $1x10^6$, $2x10^5$, $4x10^4$, $8x10^3$, $1.6x10^3$, and 320 patient PBMCs/ml, respectively. Then, 1-3 day old, HIV-1-negative, PHA-stimulated donor PBMCs were then centrifuged at 400 x g for 10 minutes at 20°C, resuspended in R-3 medium, enumerated and diluted to a concentration of $1.25x10^6$cells/ml. 0.8 ml of the donor PBMC suspension was then pipetted into six wells of two consecutive rows of a Costar (Cambridge, Massachusetts, USA) 24-well microculture plate. Thereafter, 1.0ml of patient cells from the 15ml tubes marked A through F was added to the corresponding wells of the microculture plate (see figure 4), with duplicate cultures established for each patient cell concentration. 0.2ml of R-3 medium was then added to the top row of wells. The culture was then monitored as described

above, with the viral load being calculated based on the highest dilution (i.e. least number of patient cells) yielding a positive result. This result was expressed as infectious units per million cells (IU/10^6cells).

Rapid Quantitative Culture-Based Zidovudine Resistance Assay

The above described quantitative micrococultures which were set up from each patient specimen formed the basis of our novel assay for the determination of viral susceptibility. Our resistance assay was thus based on the ACTG Quantitative Micrococulture Assay with some modifications (124,219). To the bottom row of wells in the micrococulture plate containing patient and donor PBMCs was added 0.2ml of 50μM zidovudine (Glaxo Wellcome, Research Triangle Park, North Carolina, USA). The final concentration of donor PBMCs was 1x10^6cells/ml and the final concentration of ZDV was 5μM. The plate was then covered, taped around the sides with masking tape and placed in a 5% CO_2 humidified incubator at 37 °C.

Figure 4: 24-Well Plate Format for Rapid Quantitative ZDV Resistance Assay

PBS	PBS	PBS	PBS	PBS	PBS
cont. A	cont. B	cont. C	cont. D	cont. E	cont. F
5μM A	5μM B	5μM C	5μM D	5μM E	5μM F
PBS	PBS	PBS	PBS	PBS	PBS

On day 7, 1.0ml of the culture medium was removed from the surface of each well in both the control and ZDV-containing rows and stored at -70°C in sterile 1.5ml screw cap tubes. The removed supernatant was then replaced with 0.8ml of fresh R-3 medium containing 6.25x10^5, 1-3 day old seronegative, PHA-stimulated donor PBMCs. To each well of the top row was then added 0.2ml R-3 medium and to each well of the second row was added 0.2ml 50μM ZDV. The final concentration of added PBMCs was 0.5x10^6cells/ml and the final concentration of drug in the second row of wells was 5μM.

The assay was terminated on day 14. Again, from each well was removed 1.0ml of supernatant which was frozen at -70°C. Viral replication was evaluated by measuring viral p24 antigen levels (Organon Teknika or Coulter) in culture supernatants saved on days 7 and 14. A reduction in viral replication (and thus viral

titre) in the row of wells containing ZDV compared to the parallel row of drug-free control wells was taken as a crude measure of drug susceptibility.

Standard HIV-1 Drug Susceptibility Assay

Using the viral stocks generated from the qualitative PBMC macrococulture assay, a standard HIV-1 drug susceptibility assay was performed on selected patient samples according to standardized protocols (127,212-214) to confirm the results obtained using our novel quantitative culture-based ZDV resistance assay. Prior to performing the actual susceptibility assay, the infectious titre of each patient isolate was determined. This was accomplished by titrating the virus-containing supernatant stock in a 96 well flat-bottom tissue culture plate. Seven serial four-fold dilutions, ranging from 1:16 through 1:65,536 were performed in triplicate for each patient.

For this purpose, 1-3 day old, HIV-1-negative, PHA-stimulated donor PBMCs were sedimented at 400 x g for 10 minutes at 22°C. The supernatant was removed and discarded. The PBMCs were resuspended in R-3 medium and enumerated. The suspension was adjusted to $4x10^6$cells/ml and returned to the incubator until needed. Then, 200µl of 1xPBS was added to all wells in rows A, B, F, G, and H and to the wells in columns 1, 2, and 10 to 12 in rows C to E with a multichannel pipettor (see Figure 5). 150µl of R-3 medium was then added to each well of columns 4 to 9 in rows C to E with a multichannel pipettor. The virus stock to be tested was then rapidly thawed in a water bath at 37°C until only a small crystal of ice remained. The sample was then diluted 1:12 in R-3 medium (0.1ml of virus stock was added to 1.1ml R-3 medium) in a fresh sterile 1.5ml screw cap tube, and 200µl of diluted stock was added to each well of column 3 in rows C to E. To dilute, 50µl was then transferred from column 3 to column 4 in rows C to E, using a multichannel pipettor. The pipette tips were then changed and the contents of column 4 were mixed, and 50µl of sample from column 4 was then transferred to column 5. A similar procedure was employed for column 6-9, and 50µl of solution was removed from column 9 after mixing and was discarded. Finally, 50µl of donor PBMCs (at $4x10^6$cells/ml) were added to the wells of columns 3 to 9, rows C to E, moving from right to left. The plate was then covered, taped around the sides with masking tape, and incubated at 37°C and 5% CO_2 in a humidified chamber. The final dilutions of the original viral stock in columns 3 to 9 were: 4^{-2}, 4^{-3}, 4^{-4}, 4^{-5}, 4^{-6}, 4^{-7}, and 4^{-8} cells/ml as shown in Figure 5.

Figure 5: 96-Well Plate Format for HIV-1 Viral Stock Titration

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
C	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	PBS	PBS	PBS
D	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	PBS	PBS	PBS
E	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	PBS	PBS	PBS
F	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

On day 4, the cells in the wells in rows C to E were resuspended with a multichannel pipettor. Moving from right to left across the plate, the cells were resuspended and 125µl of suspension was removed and discarded, and 150µl of fresh R-3 medium was added back to each well of rows C to E, again moving from right to left. The plate was then recovered, retaped, and returned to the incubator.

On day 7 the HIV-1 titration assay was terminated and the supernatants were tested for HIV-1 p24 antigen levels according to the manufacturer's instructions using the Organon Teknika or Coulter assay kits. This was accomplished by transferring 100µl of supernatant from the titration plate to the wells of an HIV-1 p24 antigen plate which contained 100µl of R-3 medium and 20µl of the manufacturer's disruption buffer containing Triton X-100. The p24 antigen assay was then performed and a well was scored positive if the HIV-1 p24 antigen level was ≥50pg/ml. The 50% tissue culture infectious dose ($TCID_{50}$) was then calculated using the Spearman-Karber method.

Once the $TCID_{50}$ was determined, the standard antiretroviral drug susceptibility testing assay for ZDV was performed. A series of 2X working solutions of ZDV were prepared by diluting 50µl of 1mM stock ZDV in 4.95ml R-3 medium to obtain a concentration of 10µM ZDV. The 10µM stock was then further diluted to 2µM ZDV by adding 1.0ml of 10µM ZDV to 4.0ml of R-3 medium. Further dilutions were made in a similar fashion to yield 2X stock solutions of ZDV at concentrations of 10.0µM, 2.0µM, 0.2µM, 0.02µM, and 0.002µM.

The ZDV susceptibility assay was performed in a sterile flat-bottomed 96-well plate (see figure 6). 200µl of 1xPBS was added to all wells in rows A, B, G, and H with a multi-channel pipettor and in a similar way to the wells of columns 1, 2, and 9 through 12 in rows C to F. Then, 100µl of R-3 medium was added to

the wells in rows C through F in column 3 and 100µl of 2X ZDV working solution in ascending concentration was added to the wells in rows C through F in columns 4 to 8.

Thereafter, 1-3 day old, HIV-1-negative, PHA-stimulated PBMCs were sedimented at 400 x g for 10 minutes at 22°C. The supernatant was removed and the cells resuspended in R-3 medium and enumerated. The cells were adjusted to a concentration of $4x10^6$cells/ml. To each of two sterile 15ml conical centrifuge tubes was added 1.0ml of the donor cell suspension, and 1.0ml of R-3 medium was added to one of the tubes, bringing the final concentration of cells to $2x10^6$cells/ml. The cap was secured and the tube placed in a humidified chamber at 37°C and 5% CO_2. The cells in the other tube were sedimented at 400 x g for 10 minutes and the supernatant was removed. The chosen viral stock (titrated previously) was then rapidly thawed in a water bath at 37°C until only a small crystal of ice remained. The required amount of stock was then added to the tube containing the sedimented PBMCs to make the final concentration of virus 4000 $TCID_{50}$/ml (final volume was kept to ≤1.0ml). The suspension was mixed gently and incubated at 37°C in the humidified chamber for one hour.

Following the incubation, the donor cells containing the virus stock were diluted to a final volume of 2.0ml in R-3 medium. 100µl of the infected cells were then added to each well of columns 3 through 8 in rows C to E, and 100µl of the uninfected donor PBMCs in the conical tube from the incubator were added to each well of columns 3 through 8 in row F. The final volume in each well was 200µl and the final concentration of ZDV in each well in columns 3 through 8 were 0, 0.001, 0.01, 0.1, 1.0, and 5.0µM ZDV as shown in Figure 6. The plate was then sealed and incubated at 37°C and 5% CO_2 in a humidified chamber.

Figure 6: 96-Well Plate Format for ZDV Susceptibility Testing

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
C	PBS	PBS	0	0.001	0.01	0.1	1.0	5.0	PBS	PBS	PBS	PBS
D	PBS	PBS	0	0.001	0.01	0.1	1.0	5.0	PBS	PBS	PBS	PBS
E	PBS	PBS	0	0.001	0.01	0.1	1.0	5.0	PBS	PBS	PBS	PBS
F	PBS	PBS	0	0.001	0.01	0.1	1.0	5.0	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

On day 4, the plate was removed from the incubator and examined under a microscope for any HIV-1-induced cytopathic effect. 2X ZDV working solutions were made up as on the day the experiment was set up, and 0.5ml of the working solutions were added to 0.5ml R-3 medium to give 1X working solutions. Moving from left to right across the plate, the cell suspension in the wells in rows C to F and columns 3 to 8 was mixed and 125µl was removed and discarded. 150µl of the 1X ZDV working solutions were then added back to the appropriate wells. The plate was then sealed and replaced in the humidified 37°C incubator.

On day 7, the HIV-1 drug susceptibility assay was terminated and an HIV-1 p24 antigen evaluation was performed on the culture supernatants using the Organon Teknika p24 antigen assay kit. Dilutions were prepared for the wells in rows C to E, columns 3 to 8 of the susceptibility assay plate as follows:

a. To the first six wells of rows C to H in a 96-well flat-bottomed polystyrene plate (dilution plate), 205µl of R-3 medium was added followed by 25µl of lysis buffer, using a multichannel pipettor.

b. 20µl of supernatant from each well in rows C, D and E in columns 3 to 8 of the drug susceptibility assay were then transferred to their respective rows in the dilution plate using an 8-channel pipettor (1:12.5 dilution). 20µl of cell suspension was then transferred from row C to row F, row D to row G, and row E to row H in the dilution plate following mixing using the 8-channel pipettor (1:156 dilution). The plate was then placed in a low density polyethylene bag to prevent drying of the sample by sublimation. The plate was then stored at -70°C and the HIV-1 p24 antigen assay was run within 72 hours.

Prior to the determination of the p24 antigen levels, the dilution plate was thawed at ambient temperature, and 20µl of solution from each well in rows G to H was transferred to the appropriate wells of the HIV-1 p24 antigen assay kit plate which already contained 180µl of R-3 medium (1:1560 final dilution). For some samples, a range of dilutions had to be tested until the untreated control cultures could be accurately quantified on the linear portion of the calibration curve of the assay. The 50% inhibitory concentration (IC_{50}) was then calculated, as described in the above titration experiments. An IC_{50} value of $<0.2\mu M$ was

considered to be an indication that the patient's virus was sensitive to ZDV (327) and an IC_{50} value of >1.0μM was considered to be an indication that the patient's virus was resistant to ZDV.

HIV-1 Viral Phenotype Assay (HIV-1 Syncytium-Inducing Assay)

This *in vitro* assay using MT-2 cells was used to detect syncytium-inducing (SI) variants of HIV-1 (124,209,317). The assay was performed in duplicate (for each viral isolate) in 96 well flat-bottomed tissue culture plates using the cell-free viral stocks generated from the previously grown qualitative macrococultures and quantitative micrococultures, frozen at the time of harvesting and not thawed until needed for this assay. All stocks used were previously determined to be positive for HIV-1 using the Organon Teknika p24 antigen detection kit.

Prior to their use in this assay, MT-2 cells were maintained in culture at a concentration of $1\text{-}2x10^6$cells/ml in R-10 medium [RPMI 1640, 10% heat inactivated Fetal Bovine Serum, penicillin (100units/ml)/streptomycin (100μg/ml), 2mM L-glutamine, 10mM Hepes Buffer]. The cultures were split 1:10 every 3-4 days. On day 0 of the assay, the MT-2 cells were harvested and centrifuged at 400 x g for 10 minutes. The supernatant was removed and the cells were enumerated. The cell concentration was then adjusted to $3.4x10^5$cells/ml in R-10 medium.

The configuration of the test plate is shown in figure 7, with 200μl of 1xPBS added to all the wells in rows A, D, and H, and to the wells in columns 1, 3 through 10, and 12 in row G, to the wells in columns 1 and 12 in rows B, C, and F, and to the wells in 1, 2, 11, and 12 in row E. Thereafter, 150μl of the MT-2 cell suspension was added to each duplicate sample well (numbered wells in figure 7), and to the positive and negative control wells (POS and NEG wells in figure 7), and 50μl of HIV-1-infected cell-free supernatant (to be tested) was added to the duplicate sample wells, allowing for the evaluation of 16 patient samples/plate. The positive control consisted of 50μl of a known SI virus stock while the negative control was 50μl of R-10 culture medium.

Figure 7: 96-Well Plate Format for the MT-2 HIV-1 Viral Phenotype Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	NEG	1	2	3	4	5	6	7	8	NEG	PBS
C	PBS	POS	1	2	3	4	5	6	7	8	POS	PBS
D	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
E	PBS	PBS	9	10	11	12	13	14	15	16	PBS	PBS
F	PBS	NEG	9	10	11	12	13	14	15	16	NEG	PBS
G	PBS	POS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	POS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

On days 3, 6, 9, and 12, each sample well was examined for syncytium formation. When syncytia were observed, the day of positivity was noted. Following examination of the wells, the cells in each well were resuspended and 130µl of cell suspension was removed and replaced with 150µl of R-10 medium. On day 14 the assay was terminated. If no syncytia were observed on day 14, the isolates in question were deemed to be nonsyncytium-inducing (NSI).

Plasma Viral Load Assay (NASBA®)

The NASBA Assay by Organon Teknika was used to detect plasma viral RNA levels in each patient (210,211,287). The technique is used to amplify specific RNA targets quickly using small volumes of source material of different types. The basic principles of the assay involve the isolation and purification of RNA from the plasma sample to be evaluated, the amplification of specific target sequences in the HIV-1 genome and the detection of the amplified product using chemiluminescent probes. The quantity of amplified product is measured by the quantitative reading system and calculations based on the relative amounts of the wild-type RNA and three internal standards determines the original amount of RNA in the sample. The substrate for the assay was a plasma sample taken from each patient, removed at the time of PBMC isolation and immediately frozen at -70°C in 1.0ml aliquots. The procedure itself has three technical components (as shown in Figures 8-11) (336):

A. Nucleic Acid Isolation

B. Amplification

C. Hybridization/Detection

A. Nucleic Acid Isolation

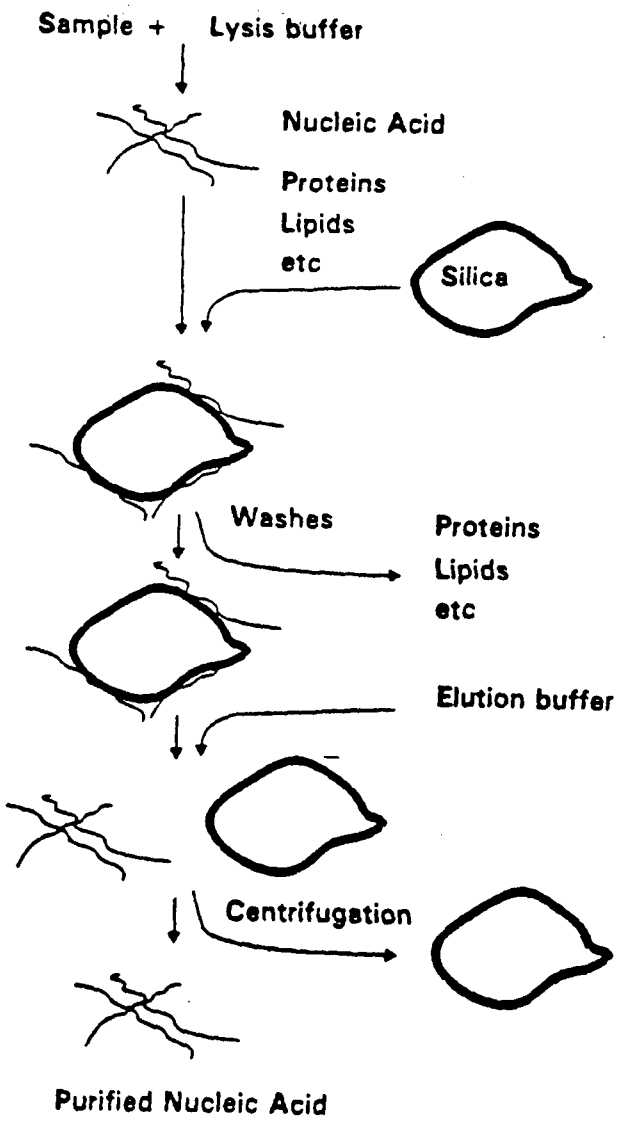
The isolation procedure is based on the binding of nucleic acid to silica particles. These silica particles remain bound to the nucleic acid through a series of washing steps. The nucleic acid is then liberated from the silica by a small volume of elution buffer. This is known as the "Boom" procedure (328). The guanidine thiocyanate (GuSCN) lysis buffer tubes, the tubes containing patient plasma samples, and the GuSCN wash buffer, were thawed at 37°C in an incubator. The elution buffer (1 mM Tris at pH 8.5) was thawed at room temperature and the Q-sphere (containing three RNA internal standards (Q_A (10^6), Q_B (10^5), and Q_c (10^4 copies))) and silica were stored at 4°C until needed. The RNA internal standards differ from the sample (wild-type) RNA and from each other by a 20-nucleotide randomized sequence with the same nucleotide composition. The standards and sample are together in the same tube, and this design of internal standards results in equal efficiency of isolation and amplification. When warmed, the lysis buffer tubes were vortexed and then "quick spun" for ten seconds to remove any liquid from the cap. In a laminar flow hood, the plasma samples were vortexed and then 100µl of plasma was added to each lysis buffer tube. The lysis buffer tubes were then vortexed, quick spun and left to sit at room temperature and the tubes with the remaining plasma were returned to the -70°C freezer.

In a separate clean (free of viral RNA) laminar flow hood, the Q-sphere was reconstituted with 220µl of elution buffer and vortexed, and 25µl of the suspension was added to 225µl of elution buffer in a separate 1.5ml screw cap tube and vortexed. On a bench top in the isolation area, 20µl of calibrator was added to each lysis buffer tube, which was then vortexed and quick spun. The silica was then vortexed and 50µl was added to each lysis buffer tube. The silica has a high density and thus had to be vortexed after every second tube. The lysis buffer tubes were then left for 10 minutes at room temperature and were vortexed every two minutes. During the 10 minutes, two 50ml conical polystyrene tubes, one containing ~30 ml 70% ethanol and the other containing ~12ml acetone were prepared. The lysis buffer tubes were then quick spun and the lysis buffer was removed. 1000µl of wash buffer was then added to each tube. After a vortex and a quick spin, the wash was removed and another 1000µl of wash buffer was added to the tubes. The vortex and quick spin were repeated and the washes were repeated (two wash buffer washes, two 70% ethanol washes and one acetone wash total). Following the final wash with acetone, the tubes were spun

for exactly ten seconds to pellet the silica. All the acetone was removed and the tubes were placed in a block heater at 56°C without their caps on, but covered with a paper towel, to dry.

The caps were then replaced and 50µl of elution buffer was added to each tube. After vortexing, the tubes were placed in the heating block at 56°C with their caps on for ten minutes, vortexing once again after 5 minutes. During the incubation time, ten new 1.5ml screw cap tubes were obtained and labeled for each sample. Following the incubation, the lysis buffer tubes were spun for 2 minutes at maximum speed in the microcentrifuge, and 5µl of supernatant was removed and transferred to the fresh screw cap tubes. The lysis buffer tubes were then stored at -70°C and the 5µl samples of purified viral RNA were frozen at -70°C until they were needed for the amplification step of the procedure.

Fig. 8: Schematic presentation
Nucleic Acid Isolation process



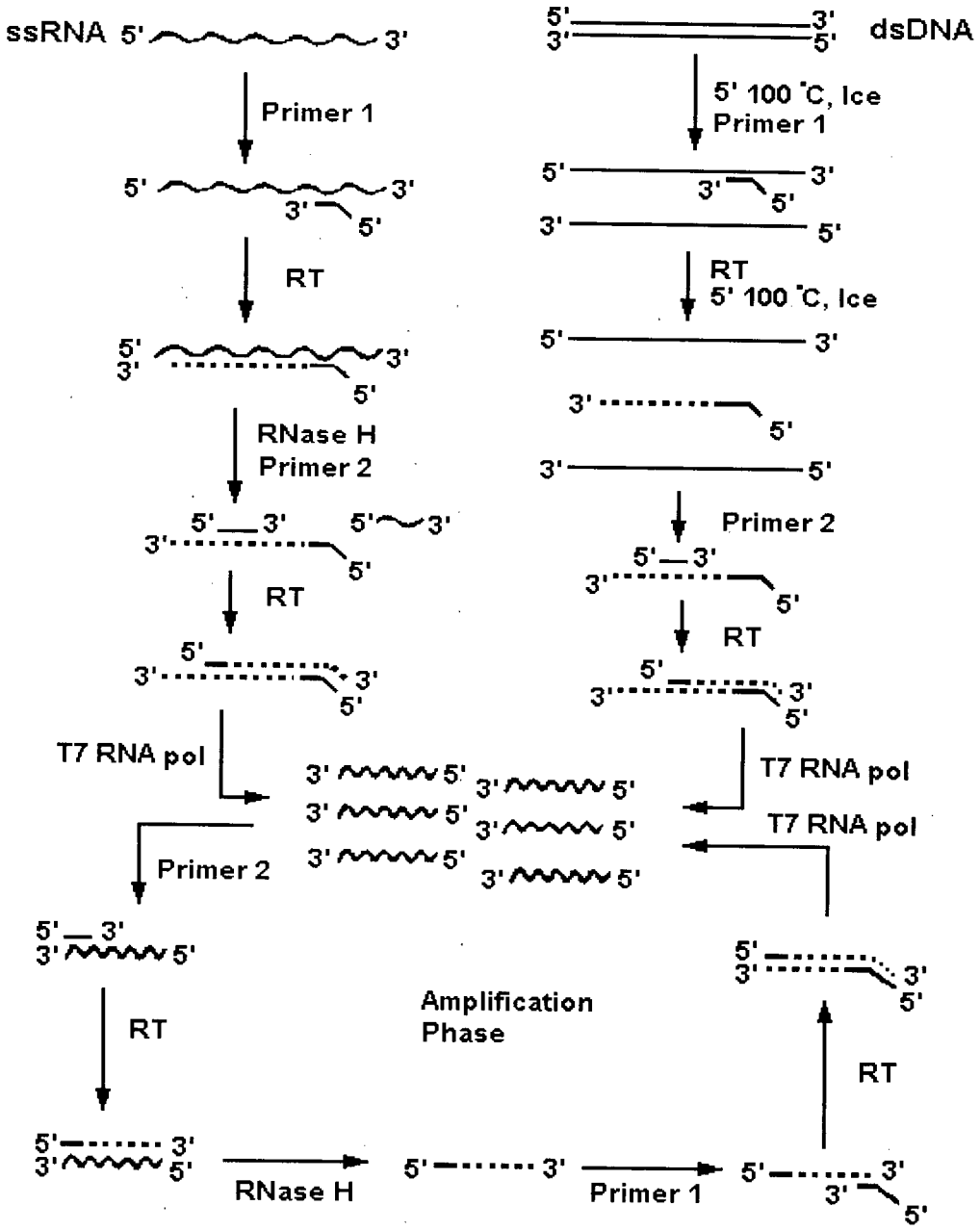
B. Amplification

The amplification reaction is based on the simultaneous action of three enzymes: Avian Myelobastosis Virus reverse transcriptase (RT), Escherichia Coli RNase H and Phage T_7 RNA polymerase. The steps of the reaction take place within one reaction tube at a constant temperature (41°C). Basically, the amplification process begins with the annealing of Primer 1 (P_1 (5'-

*AATTCTAATACGACTCACTATAGGG*ATTGCCTCTCTGCATCATTA-3')) (337), which contains a 3' terminal sequence which is complementary to the target sequence and a 5' terminal sequence which is recognized by T_7 RNA polymerase (in italics). The target sequence is a highly conserved region of the *gag* gene in the HIV-1 genome. The RT then extends P_1 to synthesize a copy of cDNA. RNAse H then degrades the RNA strand of the resulting RNA:DNA hybrid. This allows primer 2 (P_2 (5'-AGCATTGGGACCAGCGGCTA-3')) (337) to anneal to the single-stranded cDNA. Following annealing, P_2 is extended with RT to form a double-stranded DNA containing a double stranded T_7 promoter. The T_7 RNA polymerase recognizes the promoter and generates multiple copies of a single-stranded RNA product. Each copy serves as a template for a repetition of the process described above, however, the annealing of primers is reversed due to the anti-sense RNA. The amplification reaction occurs over a 90 minute time period at a constant temperature, resulting in the accumulation of single-stranded RNA target.

The procedure was begun in the clean laminar flow hood. The enzyme mix, containing the three enzymes and primer diluent (dimethylsulfoxide (DMSO) in water) were thawed. First, 45µl of enzyme diluent was added to the enzyme tube, which was shaken lightly. It was then set aside at room temperature for at least 15 minutes and no longer than 45 minutes before use. Light shaking was applied during this time to ensure adequate resuspension of the enzyme. Following this, 120µl of primer diluent was added to the primer tube, which was then vortexed vigorously to ensure complete resuspension of the primers. On a clean bench top, 10µl of primer mix was then added to each screw cap tube containing the 5µl aliquot, which had previously been removed from the -70°C freezer and thawed. The tubes were then placed in a 65°C heating block for 6 minutes, then transferred to a 41°C heating block for 6 minutes, following which, 5µl of enzyme solution was added to each tube. One tube was removed from the heating block at a time. Following the addition of the enzyme, the tube was flicked several times to ensure that the mixture was well homogenized. The tube was then replaced in the heating block. After the enzyme was added to the last tube, the tubes were then heated for an additional 5 minutes. Following the incubation, the tubes were transferred to a 41°C water bath away from the clean area and away from the isolation area and incubated for 90 minutes. Following this amplification step, the tubes were frozen at -70°C and transported to the hybridization/detection area.

Fig. 9: Amplification



C. Hybridization/Detection

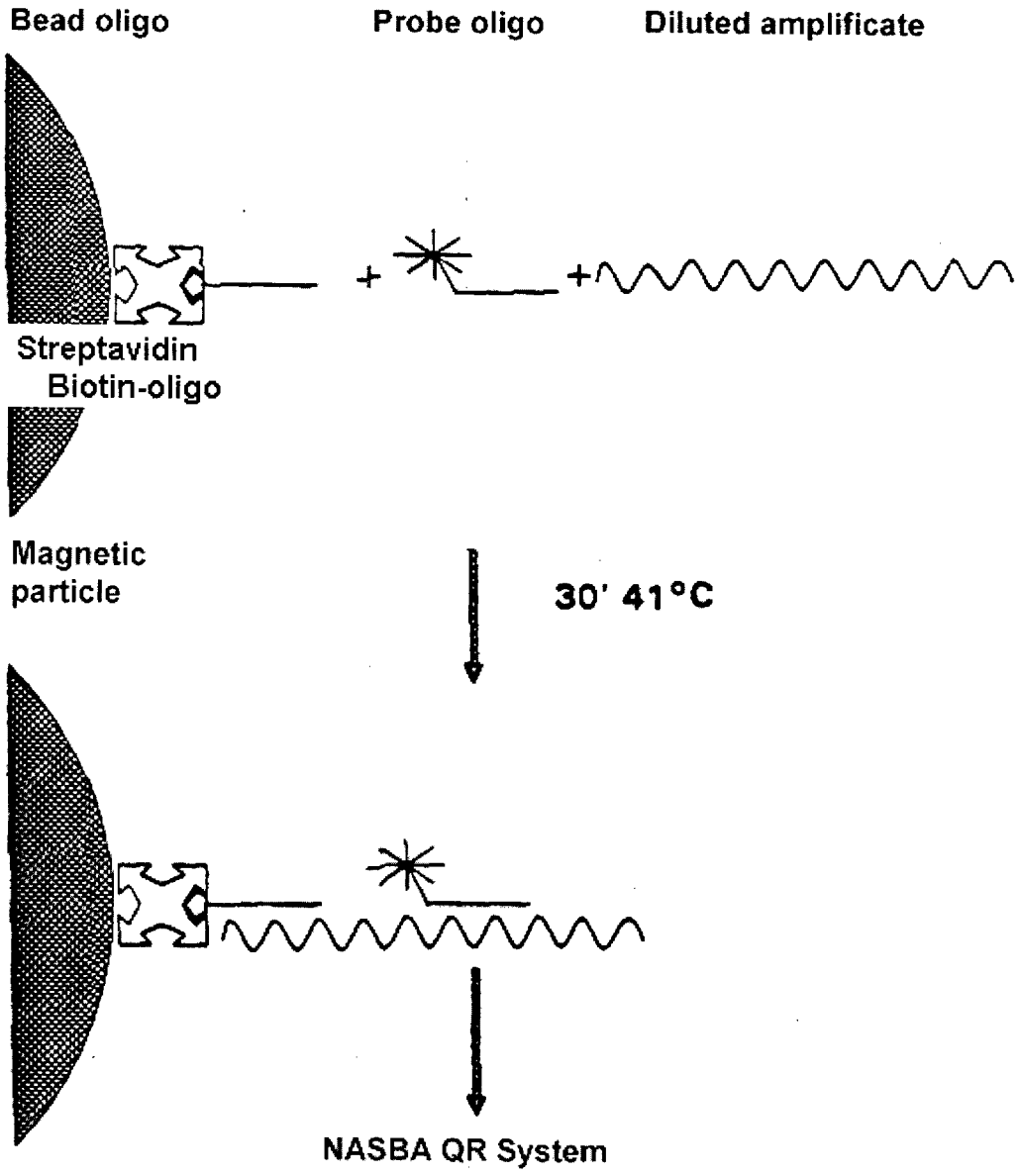
The quantitation of the amplified products is performed using the NASBA Quantitative Reading System (NASBA QR System). It is an automated system which utilizes electrochemiluminescence technology (ECL). This means that a chemical reaction takes place in an electromagnetic field and a light emission is produced. The hybridization format consists of a magnetic bead-oligo which is coated with streptavidin.

To it is bound a biotin-oligo which binds to a specific sequence on the target RNA. In addition, four ruthenium (Ru) labeled probes are used, one for each internal standard and one for the sample RNA. The probes bind to the RNA and are part of the chemical reaction that takes place during the detection.

In a dead air box, the hybridization mixtures were prepared according to the manufacturer's instructions. In the hybridization room, 41 culture tubes were obtained and arranged into three rows of ten tubes and one row of eleven tubes. With a repeat pipettor, using a 0.5 ml tip, 20µl of wild type (WT) hybridization mixture was added to each tube in the row with 11 tubes. To each of the first row of 10 tubes was added 20µl of Qa hybridization mixture. The additions were repeated for the next ten tubes with the Qb mixture and again with the Qc mixture in the last row of tubes. Qa, Qb, and Qc differ from each other only by a small, randomized amplification fragment and each hybridization mixture contains probes specific for one internal standard.

Ten eppendorf tubes were then obtained and labeled with the same identifier as the sample amplification tubes. To each tube was added 100µl of detection diluent, and 5µl of amplified product. The tubes were then vortexed and quick spun. For each sample, 5µl of diluted product was added to the first column of four culture tubes containing the WT, Qa, Qb, and Qc mixtures, 5µl of detection diluent was added to the Assay Negative Tube (the 41st tube). The tubes were then covered with adhesive tape and shaken lightly, and placed in a water bath at 41°C for 30 minutes, shaken every ten minutes.

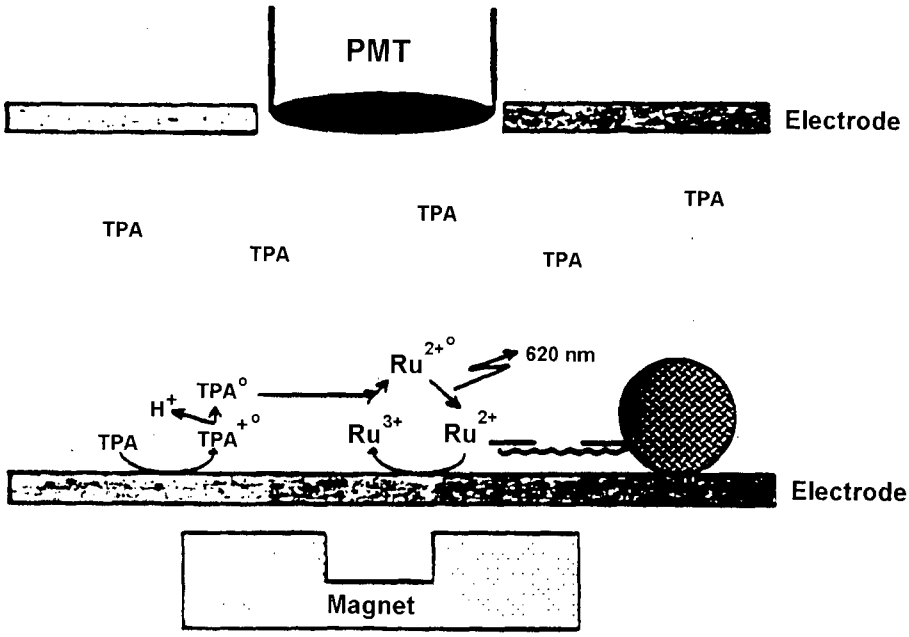
Fig. 10: Schematic Representation of the Hybridization Format



Following the hybridization, 300µl of Assay solution (containing 0.1M free Tripropylamine (TPA)) was added to each tube and to a separate clean culture tube using a repeat pipettor and a 2.5ml syringe. The detection carousel was then loaded with the culture tubes; beginning with the reference tube, the assay

negative tube and then the sample tubes, and placed into the detection machine for reading. At this point, each sample tube contains a buffer solution with free TPA and RNA product linked to a magnetic bead and to a ruthenium-labeled probe. Figure 11 illustrates the chemical reaction which then occurs in the quantitative reading system. Upon activation of the magnet, the magnetic beads are pulled against an anode by the magnetic field. A specific voltage is applied at the anode, which initiates an oxidation reaction of both the TPA and the Ru. Both molecules release an electron and become positively charged. Upon oxidation, the TPA releases a hydrogen atom (deprotonation) and becomes an unstable, highly reducing intermediate. A reaction then takes place between the TPA intermediate and the oxidized Ru. The Ru is elevated to an excited and unstable state. When the excited Ru falls back to its base state, a light photon is emitted at 620 nm. The light emission is then measured by a photo multiplier tube (PMT). The amount of light emitted is proportional to the amount of product in each tube. Calculations based on the relative amounts of the four products reveal the original amount of wild-type RNA in the sample. Plasma viral load results were expressed as copies/ml of plasma.

Fig. 11: Electrochemiluminescence Process



Viral Isolate Sequencing

The *pol* gene of selected HIV-1 isolates was sequenced to determine if ZDV resistance mutations were present at codons 215 and 219, to confirm the phenotypic results obtained using our novel quantitative culture-based ZDV susceptibility assay (215-218). The sequencing was performed in collaboration with the laboratory of Dr. Sharon Cassol (British Columbia Centre of Excellence in HIV-1/AIDS) and was based on stored frozen pellets of PBMCs isolated from each patient at the time of initial sample processing. PCR-based sequencing was completed on DNA isolated from these cell pellets.

The procedure involved two separate PCR reactions, and two sets of nested primers as previously described.

The amplified DNA was purified. The PCR product was run on an agarose gel and the resulting bands containing the appropriate DNA fragments was removed and purified using Centri-Sep columns. The DNA was then sequenced using an ABI DNA automated sequencer and the results reported as wild type or mutant for each codon of interest (215 and 219). An isolate was considered genotypically resistant if a characteristic mutation was present at one or other codon.

Clinical Data

For each patient, the following data were available: presence or absence of symptoms, presence or absence of encephalopathy, mode of transmission, and prior antiretroviral therapy (including any antiretroviral therapy received by the mother during pregnancy). This information, along with the CD4+ T cell count and percent, was integrated into our analysis of the results.

Statistical Methods

Descriptive statistics were performed for quantitative measurements of plasma viral load and cell-associated viral titre. Comparisons of these measurements with phenotype and ZDV resistance assay results were carried out using the Mann-Whitney test. Bivariate analysis of quantitative measurements of viral load and cell-associated viral load were carried out using Spearman's rank correlation coefficient. Bivariate relationships between qualitative, dichotomous variables were evaluated using Pearson's chi-square test. All p-values are two-sided.

RESULTS

Patient Population

At least one virologic parameter was evaluated in a total of 86 patients as shown in Table 5. 12 patients from Ottawa, 14 from Vancouver, 10 from Montreal Children's, 6 from London, 1 from Halifax, 12 from St. Justine's, and 31 from Toronto were assessed.

Table 5: Study Population

CITY	SITE	NUMBER
Toronto	Hospital for Sick Children	31
Vancouver	BC Children's Hospital	14
Ottawa	Children's Hospital of East	12
Montreal	Saint Justine's Hospital	12
Montreal	Montreal Children's Hospital	10
London	St. Joseph's Health Centre	6
Halifax	Izaac Walter Killan Hospital	1

Qualitative Macrococultures

Qualitative macrococultures were performed for 74 patients (see Table 6) who were enrolled in the study and viral isolates were successfully generated in 60 (81%) study participants.

Table 6: Qualitative Macrococultures

Culture	Number	Percent
Positive	60	81
Negative	14	19

Cell-Associated Viral Load (Quantitative Micrococultures)

In general, these were set up in parallel with the qualitative macrococultures, with the highest dilution of patient cells yielding a positive culture taken as a measure of the number of infectious viral units per million cells (5, 25, 125, 625, 3125, or 15625 IU/10^6 cells, respectively, based on the dilutions we have used). Table 7 shows the descriptive characteristics of the cell-associated viral loads obtained in 60 pediatric patients in which the assay was completed. The minimum load was 5 IU/10^6 cells and the

maximum load was 15625 IU/10^6 cells. The mean titre was 543 ± 2094 IU/10^6 cells, the median being 125 IU/10^6 cells. Categorically, 25% of the samples had loads below 25 IU/10^6 cells, and 75% below 125 IU/10^6 cells. A total of 13 samples yielded indeterminate results, 8 with loads below 5 IU/10^6 cells, and 5 others with apparently positive results, but an inconsistent pattern of results on serial cell dilutions. Consequently, these latter 5 values were not felt to be interpretable.

Table 7: Descriptive Characteristics of Cell-Associated Viral Load (IU/10^6 cells)

Cell-Associated Viral Load	**IU/10^6 Cells**	**Log_{10}**
Number of Samples	60	60
Minimum	5	0.70
Maximum	15625	4.19
Mean	543	1.85
Standard Deviation	2094	0.80
Coefficient of Variation	385%	43%
25th Percentile	25	1.40
Median	125	2.10
75th Percentile	125	2.10

Viral Phenotype

Viral phenotype (SI/NSI) determinations were completed in 78 patients (from whom viral isolates were generated on qualitative or quantitative cultures) with 23 (30%) containing the syncytium inducing (SI) variant of the virus and 55 (70%) the non-syncytium inducing (NSI) variant (Table 8).

Table 8: Viral Phenotype (SI/NSI) Determinations

Phenotype	Number	Percent
NSI	55	70
SI	23	30
Total	78	100

Plasma Viral Load

Plasma viral load testing was completed on a total of 85 patients. Table 9 shows the descriptive characteristics of the viral load results obtained. The lower limit of detection of this assay was 1 000 copies/ml of plasma, with any sample testing negative considered to have 1 000 copies/ml for the purposes of analysis. The maximum viral load value obtained was 5 100 000 copies/ml. The mean and standard

deviation were found to be 214 580 and 682 570 copies/ml, respectively, with 25% of the samples having viral load values below 8 500 and 75% of the samples had viral load values below 76 000 copies/ml. The median value was 25,000 copies/ml

Table 9: Descriptive Characteristics of Plasma Viral Load (copies /ml)

Viral Load	Copies/ml	Log_{10}
Number of Samples	85	85
Minimum	1 000	2.00
Maximum	5 100 000	5.71
Mean	214 580	3.44
Standard Deviation	682 570	0.83
Coefficient of Variation	318%	24%
25th Percentile	8 500	2.92
Median	25 000	3.40
75th Percentile	76 000	3.88

Standard Phenotypic Resistance Testing (ACTG)

Standard phenotypic resistance testing was completed on a total of nine patient samples. 5/9 (55%) of the samples were determined to be sensitive to ZDV and 4/9 (45%) resistant.

Quantitative Culture-Based ZDV Resistance Assay

Definitive results using our pilot assay were generated in 60 patients, with 46 (77%) found to be sensitive to ZDV and 14 (23%) resistant. In an additional 14 samples, indeterminate results were obtained, due to the viral load being too low or too high, preventing us from appreciating a difference in viral replication in the presence and absence of the drug. The results are summarized in Table 10.

Table 10: Characteristics of ZDV Resistance Using Rapid Phenotypic Resistance Testing

ZDV Resistance	Number	Percent
Sensitive	46	62
Resistant	14	19
Indeterminate	14	19
Total	**74**	**100**

Viral Isolate Sequencing (Genotypic Resistance)

As mentioned in Materials and Methods, patient PBMCs at time of isolation were pelleted and frozen to be used later for viral isolate sequencing. The RT fragments of the viral genome were amplified and sequenced to establish the presence or absence of ZDV resistance-conferring mutations at codons 215 and 219 specifically. 53 samples were completed in all. Table 11 demonstrates the characteristics of genotypic resistance in those patients assessed examining codon 215 only. For further analyses, those samples that were in a transition state, were considered resistant.

Table 11: Characteristics of ZDV Resistance Using Genotypic Analysis

ZDV Resistance	Number	Percent
Wild Type (Sensitive)	27	51
Mutant (Resistant)	22	42
Transition State	4	7
Total	**53**	**100**

CD4 Cell Counts

Table 12 shows the descriptive characteristics of the CD4+ T cell counts obtained for 70 patients at the time of their initial study evaluation. Values ranged from 0-2,402 CD4 cells/mm^3 (mean 493 ± 563). The median value was 329 cells/mm^3 with 25% below 63 and 75% below 661 cells/mm^3.

Table 12: Descriptive Characteristics of CD4 Cell Counts (cells/mm^3) and CD4 Percent

CD4 Cell Count	Cells/mm^3	Percent
Number of Samples	70	69
Minimum	0	0.0
Maximum	2 402	60.6
Mean	493	18.9
Standard Deviation	563	14.0
Coefficient of Variation	114%	74%
25th percentile	63	5.0
Median	329	19.2
75th percentile	661	29.2

Mode of Transmission

The manner in which the virus was contracted (mode of transmission) was assessed in 86 patients. Of the 69 patients for whom this information was available, 47 (68%) acquired the virus perinatally, and 22 (32%) acquired the virus non-perinatally, usually through blood transfusions.

Presence of Symptoms

This information was available in 69 patients, 57 (83%) of whom were exhibiting symptoms at time of sample collection, the remaining 12 (17%) being asymptomatic.

Encephalopathy

This information was available in 56 patients, only 8 (14%) of which showed any clinical evidence of HIV-1-associated encephalopathy.

CORRELATIVE ANALYSES

Plasma Viral Load and Cell-Associated Viral Load

We compared the plasma and cell-associated viral loads in the circulation to determine if a relationship existed between these two variables, which measure slightly different stores of HIV-1. The two were positively correlated ($r=0.37$, $p=0.004$) although not absolutely so.

Viral Load and Viral Phenotype

The relationship between plasma or cell-associated viral load and phenotype was also explored. Tables 13 and 14 show that there was no correlation between these variables.

Table 13: Relationship Between Plasma Viral Load (copies/ml) and Viral Phenotype (SI/NSI)

Plasma Viral Load	**Phenotype**		**p-value***
Descriptive Measure	**NSI(n=54)**	**SI(n=23)**	**0.956**
	Copies/ml	Copies/ml	
Minimum	1 000	1 000	
Maximum	5 100 000	450 000	
Mean	299 760	84 990	
Standard Deviation	842 590	135 070	
25th percentile	8 700	10 000	
Median	25 000	34 000	
75th percentile	91 000	97 000	

*Based on Mann-Whitney test

Table 14: Relationship Between Cell-Associated Viral Load ($IU/10^6$ cells) and Viral Phenotype (SI/NSI)

Cell-Assoc. Viral Load	**Phenotype**		**p-value***
Descriptive Measure	**NSI(n=42)**	**SI(n=18)**	**0.410**
	$IU/10^6$ cells	$IU/10^6$ cells	
Minimum	5	5	
Maximum	3125	15625	
Mean	218	1302	
Standard Deviation	503	3707	
25th percentile	25	25	
Median	125	125	
75th percentile	125	125	

*Based on Mann-Whitney test

Plasma Viral Load and CD4 Cell Count

The relationships between plasma viral load and absolute CD4 cell count and percentage were assessed in 69 patients. There were no significant relationships found between these variables (data not shown).

Plasma Viral Load and Presence of Symptoms

The relationship between plasma viral load and the presence or absence of symptoms was examined. A total of 68 samples were assessed. Table 15 shows the relationship between these two variables. No significant relationship was found.

Table 15: Relationship Between Plasma Viral Load (copies/ml) and Presence of Symptoms

Plasma Viral Load	**Presence of Symptoms**		**p-value***
Descriptive Measure	**No (n=12)**	**Yes (n=56)**	**0.281**
	Copies/ml	Copies/ml	
Minimum	1 000	1 000	
Maximum	2 000 000	5 100 000	
Mean	215 240	252 460	
Standard Deviation	576 690	786 710	
25th percentile	5 250	630	
Median	10 500	27 000	
75th percentile	31 500	103 500	

*Based on Mann-Whitney test

Plasma Viral Load and Encephalopathy

Among the symptoms, it is interesting to note that the presence of encephalopathy was highly correlated with a higher plasma viral load. (See Table 16)

Table 16: Relationship Between Plasma Viral Load (copies/ml) and Presence of Encephalopathy

Encephalopathy	**n**	**mean**	**median**	**p-value**
present	8	887 870	74 000	0.023
absent	48	76 760	16 000	

Plasma Viral Load and Mode of Transmission

The relationship between plasma viral load and mode of transmission (perinatal or non-perinatal) was also explored (Table 17). In 68 patients, there was a significant correlation between these two variables ($p=0.004$), with a significantly higher plasma viral load in children who acquired their infection perinatally.

Table 17: Relationship Between Plasma Viral Load (copies/ml) and Mode of Transmission

Plasma Viral Load	Mode of Transmission		p-value*
Descriptive Measure	**Perinatal (n=46)**	**Non-perinatal (n=22)**	**0.004**
	Copies/ml	Copies/ml	
Minimum	1 000	1 000	
Maximum	5 100 000	97 000	
Mean	352 310	19 820	
Standard Deviation	895 440	26 720	
25th percentile	10 000	3 500	
Median	34 000	11 350	
75th percentile	210 000	20 000	

*Based on Mann-Whitney test

Cell-Associated Viral Load and CD4 Cell Count

The relationship between cell-associated viral load and absolute CD4 cell count and percentage were assessed in 54 patients. There were no significant relationships between these variables.

Cell-Associated Viral Load and Presence of Symptoms

This analysis was performed in parallel with that of viral load and symptoms of HIV-1 infection. In this case no significant association was found. (See Table 18)

Table 18: Relationship Between Cell-Associated Viral Load ($IU/10^6$ cells) and Presence of Symptoms

Cell-Assoc. Viral Load	Presence of Symptoms		p-value*
Descriptive Measure	**No (n=9)**	**Yes (n=46)**	0.706
	$IU/10^6$ cells	$IU/10^6$ cells	
Minimum	5	5	
Maximum	3 125	15 625	
Mean	467	606	
Standard Deviation	1 015	2 354	
25th percentile	25	25	
Median	125	125	
75th percentile	125	125	

*Based on Mann-Whitney test

Cell-Associated Viral Load and Presence of Encephalopathy

Among the symptoms, children without encephalopathy tended to have a slightly higher cell-associated viral load (Table 19), although this association did not quite reach statistical significance. It is interesting

to note that this relationship is inverted compared to the previously described relationship between plasma viral load and encephalopathy.

Table 19: Relationship Between Cell-Associated Viral Load ($IU/10^6$ cells) and Presence of Encephalopathy

Cell-Assoc. Viral Load	Presence of Encephalopathy		p-value*
Descriptive Measure	No (n=38)	Yes (n=6)	0.068
	$IU/10^6$ cells	$IU/10^6$ cells	
Minimum	5	5	
Maximum	15 625	3 125	
Mean	688	775	
Standard Deviation	2 586	1 181	
25th percentile	25	125	
Median	25	375	
75th percentile	125	625	

*Based on Mann-Whitney test

Cell-Associated Viral Load and Mode of Transmission

In contrast, a significantly higher cell-associated viral load was measured in children with non-perinatal acquisition of HIV-1 infection (Table 20). This finding also differs somewhat from the demonstrated correlation between plasma viral load and perinatal HIV-1 infection.

Table 20: Relationship Between Cell-Associated Viral Load ($IU/10^6$ cells) and Mode of Transmission

Cell-Assoc. Viral Load	Mode of Transmission		p-value*
Descriptive Measure	Perinatal (n=39)	Non-perinatal (n=16)	0.001
	$IU/10^6$ cells	$IU/10^6$ cells	
Minimum	5	5	
Maximum	3 125	15 625	
Mean	411	1 004	
Standard Deviation	823	3 899	
25th percentile	25	5	
Median	125	25	
75th percentile	625	25	

*Based on Mann-Whitney test

Comparative Resistance: Phenotype vs. Genotype

We compared the prevalence of ZDV resistance obtained using our rapid phenotypic resistance testing method and those obtained by formal sequencing (genotypic resistance). Table 21 shows the relationship between these two variables. An isolate was deemed genotypically resistant to ZDV if it carried the typical mutation at codon 215. It should be noted that a mutation, which confers resistance to a drug, would be

detectable before the virus population in a patient becomes sufficiently resistant to the drug for it to be noticed phenotypically. That may be the case with the 8 samples, which were found to have mutant codons at position 215 yet were found to be sensitive by our rapid phenotypic resistance assay. In the case of the two samples which were found to be resistant by our assay, but were wild type at codon 215, mutations at other codons may have been present which would have also conferred resistance to ZDV. It is known, in fact, that the two samples were mutant at codon 219. Thus, of 45 evaluable isolates, 35 (78%) produced concordant results, with 8/10 mismatches possibly attributable to the presence of genotypic resistance in advance of the development of phenotypic resistance.

Table 21: Relationship Between Genotypic and Phenotypic resistance

		Phenotype	
		Sensitive	**Resistant**
Genotype	**Wild Type (Sensitive)**	25	2
	Mutant (Resistant)	8	10

Comparative Resistance: Genotype vs. Rapid Phenotype vs. Standard Phenotype (ACTG)

Standard phenotypic resistance assays according to the AIDS Clinical Trials Group (ACTG) protocols were performed on selected samples to confirm the results obtained from our pilot assay. In total, assays were completed on 9 patients. Table 22 shows a comparison of the results obtained for those samples on which all three resistance assays were completed. Looking at the results from the rapid and standard phenotypic testing only, the first four results were completely concurrent between the two phenotypic assays. The fifth sample was indeterminate by our assay, susceptible by the ACTG assay, but shown to be in a transition state between wild type and mutant at codon 215 by genotypic analysis. The sixth sample, also in a transition state, was sensitive by our assay and resistant by the ACTG assay. The last three samples showed concurrence between the genotypic assay and ACTG assay, but were indeterminate or sensitive by the rapid pilot assay.

Table 22: Comparison of Genotypic, Rapid and Standard Phenotypic Resistance

Genotypic Assay	Rapid Phenotypic Assay	Standard Phenotypic Assay (ACTG)
-	S	0.088
-	S	0.144
WT	S	0.171
MU	S	0.173
TS	I	0.163
TS	S	2.65
MU	I	4.35
MU	S	>10
MU	I	>10

Genotypic Resistance and Other Variables

The relationship between genotypic ZDV resistance and absolute CD4 cell counts, CD4 cell percentages, symptoms, encephalopathy and the mode of transmission were evaluated (Tables 23-27). CD4 cell counts and percentages were lower in patients with resistant isolates. No other correlations were identified.

Table 23: Relationship Between CD4 Cell Count (cells/mm^3) and Genotypic Resistance

CD4 Cell Count	Genotypic Resistance		
Descriptive Measure	Sensitive (n=25)	Resistant (n=24)	p-value*
Minimum	11	1	<0.001
Maximum	1 900	1 806	
Mean	798	278	
Standard Deviation	574	450	
25th percentile	400	35	
Median	661	75	
75th percentile	1 080	326	

* Based on Mann-Whitney test

Table 24. Relationship Between CD4 Percent and Genotypic Resistance

Percent CD4	Genotypic Resistance		
Descriptive Measure	Sensitive (n=25)	Resistant (n=24)	p-value*
Minimum	1	0.30	<0.001
Maximum	60.6	42.0	
Mean	26.0	12.2	
Standard Deviation	13.3	11.5	
25th percentile	21.1	2.3	
Median	29	7.0	
75th percentile	32.1	21.0	

* Based on Mann-Whitney test

Table 25. Relationship Between Genotypic Resistance and Presence of Symptoms

	Genotypic Resistance		
Presence of Symptoms	**Sensitive**	**Resistant**	**p-value***
No	7 (29%)	2 (8%)	0.056
Yes	17 (71%)	23 (92%)	
Total	24 (100%)	25 (100%)	

* Based on chi-squared test (uncorrected)

Table 26. Relationship Between Genotypic Resistance and Presence of Encephalopathy

	Genotypic Resistance		
Encephalopathy	**Sensitive**	**Resistant**	**p-value***
present	2 (11%)	5 (23%)	0.301
absent	17 (89%)	17 (77%)	
Total	19 (100%)	22 (100%)	

* Based on chi-squared test (uncorrected)

Table 27. Relationship Between Genotypic Resistance and Mode of Transmission

	Genotypic Resistance		
Mode of Transmission	**Sensitive**	**Resistant**	**p-value***
Perinatal	19 (76%)	18 (72%)	0.747
Non-perinatal	6 (24%)	7 (28%)	
Total	25 (100%)	25 (100%)	

* Based on chi-squared test (uncorrected)

Phenotypic Resistance and Other Variables

Similar analyses were performed based on the phenotypic resistance data, using the rapid pilot assay (Tables 28-32). Similar results were obtained.

Table 28. Relationship Between Phenotypic Resistance and CD4 Cell Count (cells/mm^3)

CD4 Cell Count	**Phenotypic Resistance**		
Descriptive Measure	**Sensitive (n=43)**	**Resistant (n=11)**	**p-value***
Minimum	0	10	0.009
Maximum	2 402	600	
Mean	639	145	
Standard Deviation	633	181	
25th percentile	80	14	
Median	489	69	
75th percentile	912	240	

* Based on Mann-Whitney test

Table 29. Relationship Between Phenotypic Resistance and CD4 Percent

Percent CD4	Phenotypic Resistance		
Descriptive Measure	**Sensitive (n=43)**	**Resistant (n=11)**	**p-value***
Minimum	0.00	1.7	0.026
Maximum	60.0	23.0	
Mean	21.5	10.7	
Standard Deviation	14.7	8.6	
25th percentile	8.0	2.3	
Median	24.1	6.0	
75th percentile	32.0	19.4	

* Based on Mann-Whitney test

Table 30. Relationship Between Phenotypic Resistance and Presence of Symptoms

	Phenotypic Resistance		
Presence of Symptoms	**Sensitive**	**Resistant**	**p-value***
No	9 (21%)	0 (0%)	0.083
Yes	34 (79%)	12 (100%)	
Total	43 (100%)	12 (100%)	

* Based on chi-squared test (uncorrected)

Table 31. Relationship Between Phenotypic Resistance and Presence of Encephalopathy

	Phenotypic Resistance		
Encephalopathy	**Sensitive**	**Resistant**	**p-value***
Present	4 (12%)	2 (18%)	0.612
Absent	29 (88%)	9 (82%)	
Total	33 (100%)	11 (100%)	

* Based on chi-squared test (uncorrected)

Table 32. Relationship Between Phenotypic Resistance and Mode of Transmission

	Phenotypic Resistance		
Mode of Transmission	**Sensitive**	**Resistant**	**p-value***
Perinatal	30 (70%)	9 (75%)	0.724
Non-perinatal	13 (30%)	3 (25%)	
Total	43 (100%)	12 (100%)	

* Based on chi-squared test (uncorrected)

Phenotypic Resistance and Plasma Viral Load

We also examined whether any relationship existed between phenotypic resistance and plasma or cell associated viral load (Tables 33 and 34). No such relationships were identified.

Table 33. Relationship Between Plasma Viral Load (copies/ml) and Phenotypic Resistance

Plasma Viral Load	ZDV Resistance		p-value*
Descriptive Measure	**Sensitive (n=45)**	**Resistant (n=14)**	**0.487**
	Copies/ml	Copies/ml	
Minimum	1 000	1 100	
Maximum	2 400 000	5 100 000	
Mean	240 390	439 820	
Standard Deviation	562 870	1 346 610	
25th percentile	6 400	20 000	
Median	23 000	33 000	
75th percentile	110 000	76 000	

*Based on Mann-Whitney test

Phenotypic Resistance and Cell-Associated Viral Load

A comparison between cell-associated viral load and rapid phenotypic ZDV resistance was also performed. A total of 56 samples were compared. Table 34 shows the comparison between these two variables. No correlation was found.

Table 34. Relationship Between Cell-associated Viral Load (IU/10^6 cells) and Phenotypic Resistance

Cell-Assoc. Viral Load	ZDV Resistance		p-value*
Descriptive Measure	**Sensitive (n=46)**	**Resistant (n=14)**	0.248
	IU/10^6 cells	IU/10^6 cells	
Minimum	5	5	
Maximum	15 625	3 125	
Mean	584	409	
Standard Deviation	2 356	817	
25th percentile	25	25	
Median	125	125	
75th percentile	125	625	

*Based on Mann-Whitney test

Viral Phenotype and CD4 Cell Count

Finally, we were able to demonstrate a clear association between viral phenotype and absolute CD4 cell count and percentage (Tables 35 and 36), but with no other clinical variables (data not shown).

Table 35. Relationship Between Viral Phenotype (SI/NSI) and CD4 Cell Counts (cells/mm^3)

CD4 Cell Count	Viral Phenotype		
Descriptive Measure	**NSI (n=46)**	**SI (n=21)**	**p-value***
Minimum	1	0	0.001
Maximum	2 402	825	
Mean	657	169	
Standard Deviation	620	206	
25th percentile	102	56	
Median	509	85	
75th percentile	912	189	

* Based on Mann-Whitney test

Viral Phenotype and CD4 Percent

As with CD4 cell count, CD4 cell percent was compared with the viral phenotype (SI/NSI) of patient samples to discover relationships that may have existed. As with CD4 cell count, a relationship was found between CD4 cell percent and viral phenotype. A total of 66 samples had data available on both these variables. See Table 36.

Table 36. Relationship Between Viral Phenotype (SI/NSI) and CD4 Percent

Percent CD4	Viral Phenotype		
Descriptive Measure	**NSI (n=46)**	**SI (n=20)**	**p-value***
Minimum	0.3	0.00	<0.001
Maximum	60.6	30.0	
Mean	22.7	10.1	
Standard Deviation	14.1	9.2	
Coefficient of Variation	62%	92%	
25th percentile	11.2	2.7	
Median	24.5	6.4	
75th percentile	32.2	18.5	

*Based on Mann-Whitney test

DISCUSSION

Conduct of Study

This was an extended cross-sectional study, performed in all tertiary care institutions in Canada providing care for HIV-1-infected children. As the vast majority of such children are followed in one of these institutions, this can be taken as a very representative evaluation of the status of pediatric HIV infection in our country between 1993-95. Standardized case report forms were designed to ensure a uniform method for the collection of clinical data. In addition, all of the blood samples were provided to the virology research laboratory in a blinded fashion, and the results of all assays were completed and the results tabulated individually before any analysis was undertaken. We are confident that this methodology has led to the generation of a very reliable and objective data set, increasing the power of the results we have generated.

Cell-Associated Viral Load

Cell-associated viral load was determined from the quantitative micrococultures that were set up according to standard protocols. These assays were completed in 60 patients. Cell-associated viral load was highly significantly correlated with plasma viral load (r^2=0.37, p=0.004). Sei et al also found this association (r^2=0.86, p=0.0025) (323) in 16 patients with symptomatic HIV-1 infection. In our study, cell-associated viral load was also significantly correlated with mode of transmission (p=0.001), higher following perinatal infection. A trend toward viral load being associated with the presence of encephalopathy was seen, (p value approaching statistical significance), suggesting an association between viral load and this most severe complication of pediatric HIV-1 infection.

Plasma Viral Load

Plasma viral load was measured using the Organon Teknika NASBA assay. This assay was chosen because it only required a small volume (100μl) of plasma, a particularly favorable characteristic in pediatric studies. Duplicate samples were not required because, according to the manufacturer, good precision (<0.3 logs) is produced by the use of the three internal RNA calibrators (328). The threshold of

detection of this assay was 1000 copies/ml of plasma. Overall, this assay generated results in one day and 10 samples could be run in one assay. The RNA isolation procedure was a bit tedious, but the remainder of the assay was highly mechanized and efficient, especially since the amplification segment of the assay was isothermal.

A total of 85 samples were evaluated for plasma viral load, generating results highly correlated with the cell-associated viral loads. However, a highly significant correlation was found between plasma viral load and mode of transmission ($p=0.004$), favouring higher loads in non-perinatal infection. Saag et al. (20) also found that each of five children infected with HIV-1 *in utero* or during the perinatal period had detectable plasma viremia, independent of CD4+ cell count, duration of infection, or clinical state. However, the 4 children infected by HIV-1 at older ages had detectable plasma viremia less frequently. The reasons for this association merit further exploration, but may relate to treatment effects not controlled for in our current analysis. As such, it may be that patients receiving antiviral drugs may have lower plasma viral loads. If this were more frequent in patients with perinatal acquisition of infection, this could explain our results. Cell-associated viral load is much less susceptible to treatment effects and the true relationship between viral load and mode of acquisition may be towards higher loads following perinatal infection, as shown in the cellular assays. This is relatively easy to explain, as such patients would have been infected longer and there may be an association between total body viral burden and the duration of infection. The consistency of the pathologic significance of viral burden may best be appreciated by examining the relationship of plasma and cell-associated viral load with encephalopathy, a serious virologically-related complication of HIV-1 infection in children.

Phenotypic Resistance

Phenotypic resistance to ZDV was not found to be associated with plasma viral load, cell-associated viral load, phenotype, and mode of transmission or presence of encephalopathy. However, a strong correlation was seen between ZDV resistance and lower absolute CD4+ T cell count ($p=0.009$) and percent ($p=0.026$). This trend has been demonstrated on numerous occasions in the past, and speaks of the more rapid acquisition of ZDV resistance in patients with advanced immune disease, first described by Richman et al. in 1991 (217).

Genotypic Resistance

Genotypic resistance was not found to be associated with mode of transmission or presence of encephalopathy. However, as with phenotypic resistance, a strong correlation was found between genotypic resistance and lower absolute CD4+ T cell count ($p<0.001$) and percent ($p<0.001$). This confirms the previously demonstrated associations with phenotypic drug resistance.

Prevalence of Zidovudine Resistance

In this study, using our rapid phenotypic resistance assay, the prevalence of ZDV resistance in the population was 19% (14/74). There were 46/74 (62%) sensitive isolates while indeterminate results were obtained in 14 (19%) cases. Examining definite results only, 77% (46/60) of the virus isolates were susceptible to ZDV and 23% (14/60) were resistant. Using the genotypic resistance assay, 51% (27/53) of the isolates sequenced showed the presence of a mutation conferring resistance to ZDV at codon 215, and 49% (26/53) did not. The higher rate of genotypic vs. phenotypic resistance reflects the fact that resistance mutations appear in the viral population long before the development of full-blown phenotypic resistance. For our genotypic studies, we focused on changes at codon 215 of the reverse transcriptase gene. In previously published studies, every case where a reduction in ZDV susceptibility occurred, there were mutations in the reverse transcriptase at codon 70, codon 215 or both (216). A complete picture could have been generated by looking at changes at codon 70. However, we were not prepared to undertake this additional sequencing as part of this pilot project.

Using the formal standard phenotypic resistance assay (124), which we conducted to confirm the results of our rapid phenotypic assay, only nine useable results were obtained. We attempted to complete the test in 29 cases. The high failure rate speaks to the complexity of the assay and its lack of applicability to the evaluation of small samples, such as those generated in pediatric studies. It should be noted that concurrence between the two phenotypic assays was only obtained for strains that were sensitive by the rapid pilot test we are developing.

The rapid assay has several advantages. It can be adapted to test susceptibility of HIV-1 to other antiretroviral drugs. It also takes much less time than the standard phenotypic assay. It directly cultures virus-containing patient PBMCs, immediately following their isolation from whole blood. Virus stocks do

not need to be generated and then titrated. With the standard phenotypic assay, virus stocks do need to be generated, which may or may not be successful, especially if small volumes of blood are used. Because there are so many more steps involved in the ACTG resistance assay, there is more room for error and failure. For example, 60 rapid phenotypic resistance assays could be performed, whereas only 29 titrations were successfully performed and of those 29, only 11 generated titres high enough that the second part of the resistance assay could be performed. The fewer the steps involved, the more results can be generated more easily. Also, for treatment purposes, a test which can detect resistance quickly is needed, so that decisions can be made regarding changes in therapy as soon as possible.

One disadvantage of the rapid resistance assay is that in the limited number of samples we evaluated, it seemed to underestimate the prevalence of resistance. More comparative data will have to be generated, although we did detect a significant number of resistant isolates quite apart from the comparisons and we are encouraged by the preliminary results of Nelson (300) using an assay similar to ours. Clearly, more work is needed, but we feel that a rapid phenotypic assay will become the standard of care in the near future.

Viral Phenotype

Debate still exists as to the clinical usefulness of evaluating viral phenotype in HIV-1-infected patients. It has been shown that patients harboring SI virus are more likely to progress to AIDS more rapidly. Nielsen et al. have also shown that development of phenotypic and genotypic resistance was faster in patients harboring SI isolates (326). In this study, viral phenotype was determined in a total of 78 patients. 23/78 (29%) of the patient isolates had the SI phenotype while 55/78 (71%) did not. There was no correlation between viral phenotype and plasma viral load, cell-associated viral load, phenotypic resistance, and presence of symptoms, or mode of transmission. There was also a strong correlation between viral phenotype and absolute CD4+ T cell count ($p=0.001$) and percent ($p<0.001$), confirming previous observations. The independent value of measuring the phenotype was not demonstrated in this study and remains to be fully elucidated.

CONCLUSION

We have successfully completed a comprehensive cross-sectional study of pediatric HIV infection in Canada. This, in itself was a very useful exercise. It has led to the creation of a truly national database, useful to all researchers in this field. It has also established an infrastructure that could be used for the design and implementation of future clinical trials and other studies of pathogenesis.

We have detected a high prevalence of zidovudine resistance, a fact which will have to be taken into account in the design of future guidelines and trials. In establishing our "viral inventory", it is somewhat surprising that we were not able to demonstrate any significant associations between different parameters, except for the relationship between plasma and cell-associated viral load. It may be that the associations will become more clear as prospective data are collected. Preliminary data to support this statement may be the association between viral load and mode of transmission, which may be reflective of the higher loads seen in children that have been infected for a longer period of time. Whatever the answer, we have successfully defined the status of HIV-1-infected Canadian children, and future students and other researchers will be able to build on this to increase our insight into the ideal management of these individuals.

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