VIRAL LOAD IN VANCOUVER

A Report from the 11th International Conference on AIDS
Vancouver, British Columbia, 8-11 July 1996

(Version 1.0)

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The Treatment Action Group (TAG) fights to find a cure for AIDS and to ensure that all people living with HIV receive the necessary treatment, care and information they need to save their lives. TAG focuses on the AIDS research effort, both public and private, the drug development process, and our nation's health care delivery systems. We meet with researchers, pharmaceutical companies and government officials, and resort when necessary to acts of civil disobedience, or to acts of Congress. We strive to develop the scientific and political expertise needed to transform policy. TAG is committed to working for and with all communities affected by HIV.

Acknowledgements. Thanks to the Health Care Communications Group (HCG) and Bruce Polsky, M.D., of Memorial Sloan-Kettering Cancer Center, for asking me to report on viral load in Vancouver. Thanks as well to Donald Abrams, David Chernoff, Spencer Cox, Wafaa El-Sadr, Gregg Gonsalves, Anthony Fauci, David Ho, Don Kotler, Cliff Lane, Martin Markowitz, Jim Neaton, Vincent Pieribone, Theo Smart, John Sninsky, Joseph Sonnabend and the TAG board, staff and membership for stimulating discussions. Finally, thanks to the support of Michael Palm, the Royal S. Marks Foundation Fund, The Red Hot Organisation, Mathilde Krim, Ph.D., Marvin Shulman, and our many other dedicated supporters, without whom this report and TAG's work, would not be possible.

Any additions, emendations, corrections, errors, omissions, opinions, questions or responses may be e-mailed to the author at alacran7@aol.com or faxed to TAG at 212.260.8561.

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This report is dedicated to the memory of Carl Parisi

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After a scientific revolution many old measurements and manipulations become irrelevant and are replaced by others instead.

-- Thomas S. Kuhn
The Structure of Scientific Revolutions
1962/1970, p. 129

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INTRODUCTION

The year 1996 has brought a sea change in AIDS research and treatment. Three major factors have contributed to this sea change: a new understanding of viral pathogenesis, new and powerful antiretroviral treatment regimens, and new, more powerful tools for measuring HIV levels in the blood and elsewhere in the body. Indeed, it was due to the sensitivity of these new viral load assays that researchers were able to determine the kinetics of HIV replication and immune system clearance within the infected human host, and devise new therapeutic approaches to reduce viral replication. The impact of the viral load assays on HIV pathogenesis and treatment research can be compared to the impact of the Hubble Space Telescope on cosmology: both allowed researchers to see their subject with unprecedented resolution.

Previous methods of quantifying viral activity were cruder and much less sensitive than the new viral load assays, most of which measure HIV ribonucleic acid (RNA) in the blood. Originally, HIV infection was diagnosed indirectly, by detecting antibodies with ELISA or Western Blot tests. Later, several companies developed p24 antigen capture assays to measure blood levels of the viral capsid protein p24. Unfortunately, p24 levels correlated poorly with progression; many people progressed to AIDS and died without any detectable p24 in their blood. Whatever p24 they had was bound to anti-p24 antibodies and therefore undetectable. Subsequently, immune-complex dissociated (ICD) p24 assays were developed to address this problem, but p24 remained an insensitive marker for viral replication.

Treatment research was stymied by the limitations of previous tests. Some researchers used p24 to monitor treatment effects, while others used quantitative co-cultures, putting blood cells from HIV-infected persons into culture with T cell lines and counting the number of p24 particles which emerged. Like p24 antigen tests, however, quantitative microculture techniques were limited by their lack of sensitivity; virus could be cultured from less than 50% of patients with more than 200 CD4 cells/mm^3 (Saag 1996). Most common of all was the use of the peripheral blood CD4+ T lymphocyte count to assess treatment effects. While CD4+ T cell levels give an accurate picture of the degree of immune destruction, and provide valuable short-term prognostic information on a given individual's risk of progression and death, they are highly variable and do not give a direct picture of the level of viral activity.

After the XI International Conference on AIDS in Vancouver, British Columbia, on 8-11 July, 1996, it is clear that monitoring of plasma HIV RNA levels will become a standard tool for diagnosis, prognosis and treatment of HIV infection and for the medical monitoring of antiretroviral therapy. CD4+ T cell monitoring remains an important parameter, especially in more immune-compromised persons, but viral load will be a key determinant in initiating and changing treatment regimens.

Unfortunately, while its usefulness is clear, how best to use viral load monitoring is still far from obvious, and there are several competing approaches to controlling viral load, which must be rigorously compared. Moreover, the addition of viral load monitoring adds to the already swelling

costs of HIV care, and the necessary frequency of testing is far from clear. Finally, not all the tests are yet FDA-approved, and the cost of the one approved thus far, Roche's Amplicor™ PCR test, is $150 per test in the USA (while it is less than half that in Canada). Perhaps FDA approval of competing tests will impose competitive pressure on Roche to lower its price.

This report is an interim attempt to survey the rapidly-evolving field of research using viral load tests for the diagnosis, prognosis and treatment of HIV infection, to determine what is known and to pose some questions about what remains to be determined. References in the text are generally to abstracts from the XI International Conference on AIDS, which can be accessed on the Internet through the American Medical Association (AMA) homepage at http:\\www.ama-assn.org. Health Care Communications Group (HCG) provides access to the AMA HIV homepage and to daily conference summaries at http:\\www.healthcg.com.

EXECUTIVE SUMMARY

Viral Load Assays. Recently, several very powerful and sensitive tests for measuring the presence of HIV RNA in the blood have become available to researchers and to some clinicians and patients whose insurance covers their cost. The FDA has approved one viral load test, the Roche Amplicor™ reverse transcriptase polymerase chain reaction (RT PCR) test, for diagnostic and prognostic use. Two other tests, Chiron's Quantiplex™ branched-chain DNA (bDNA) test and Organon Teknika's nucleic acid sequence-based amplification assay (NASBA) are also available, though not yet FDA approved. The Roche test can detect down to about 200 RNA copies per cubic milliliter (mm^3); the Chiron and Organon tests detect to about 500 copies. New, more sensitive bDNA and PCR research assays can detect virus to a lower limit of around 25 copies/mm^3. Because different tests differ in their sensitivities, the same viral load test should be used in a given individual over time. In the USA, Roche's Amplicor costs $150 per test, while in Canada it costs $63.

Viral Load & HIV Pathogenesis. In 1995, David Ho and George Shaw revolutionized HIV research by showing that HIV replicates at an astonishing rate, with up to 10 billion HIV copies being made per day, and an equal number being removed by the immune system. Each milliliter of blood contained, on average, 60,000 HIV RNA copies, of which most were defective, replication-incompetent virus. Using Abbott's protease inhibitor ABT-538 (now Norvir™ brand ritonavir), Ho showed that the half-life for immune clearance of HIV was about 1.25 days, and the half-life of infected CD4+ T cells was similar (Ho 1995, Wei 1995, Perelson 1996). This discovery paved the way for new, more aggressive antiretroviral treatment strategies which are now bearing fruit. Early in HIV infection, virus production and immune system clearance lead to an apparent, but deceptive "steady-state," when virus levels and CD4+ T cell levels appear to be in balance. Actually, the immune system is undertaking a heroic, but ultimately unsuccessful, effort to contain viral replication. At the time, drug therapy appeared capable of reducing the viral load by 50% or more, but unable to reduce viral levels below limits of detection. This would all change in 1996. In Vancouver, five studies using various combination anti-HIV regimens confirmed Dr. Ho's findings on the kinetics of viral and T cell replication, and demonstrated a two-phase viral decay slope, with a 90-99% reduction in the first two weeks of therapy, and a slower second-phase decline to undetectable viral levels over the next 12-24 weeks, reflecting clearance of chronically-infected T cells and macrophages (Ho Th.B.930).

Viral Load & Diagnosis. HIV RNA tests are useful for confirming a positive ELISA or Western Blot test, for diagnosing acute HIV infection before antibody seroconversion has occurred, and for the early diagnosis of HIV-infected infants (Brown Tu.B.2374, Busch Tu.A.153). However, the Roche Amplicor™ test is much less sensitive than NASBA, and somewhat less sensitive than bDNA, in diagnosing HIV strains which differ from the subtype B strains common in Europe and North America. NASBA is better for detecting subtype E, common in Thailand, and subtypes A, F and G. Roche is working on modified PCR tests to better detect other HIV strains. None of the assays detects HIV strains from group O. Such strains are increasingly common around the world and have begun to appear in the USA (Sullivan Th.A.922, Artenstein *JAMA* 1996).

Viral Load & Prognosis. In Vancouver, multiple lines of evidence converged to demonstrate that viral load was clearly of great prognostic value in assessing a given HIV-infected individual's risk of progression and death over a two-, five- and ten-year period. The most compelling evidence was presented by John Mellors in a retrospective survey of 1,601 men from the Multicenter AIDS Cohort Study (MACS) using viral RNA levels in 1985 to predict clinical outcome in 1995 (Mellors Mo.B.533, We.B.410). Baseline HIV RNA was the most predictive factor for progression and survival in the MACS cohort. For 855 patients who died of AIDS the baseline RNA count was 24,200; for 993 who developed AIDS it was 19,145; for 749 who remain alive it was 4,426 and for those who did not develop AIDS it was 3,636. Those entering with viral load over 30,000/^{ml} had a 13-fold increased relative risk of AIDS and an 18-fold increased risk of death. Risk of progression and death grows steadily with increasing viral load. CD4 remained a useful predictor, especially for patients with lower CD4 cells. Mellors stated that viral load and CD4 should be used together in assessing prognosis, and cautioned that RNA levels in the MACS cohort were probably 30% lower than with contemporary testing because the MACS used heparinized blood samples. Six other papers confirmed the association between higher viral load and more rapid progression in adults and children (Graham We.B.411, Mofenson We.B.315, Rakusan We.B.3327, Shearer Th.B.910, Weverling Th.B.4330, Yerly We.B.413), though CD4 levels, particularly when low, remained valuable prognostically over shorter periods of time (Popescu Th.B.4298). Long-term non-progressors have lower viral load than rapid progressors, both in adult and in pediatric populations, and among long-term survivors with low CD4 counts, those with lower viral load remain healthier than those with higher viral load (Lefrère Mo.B.1380, O'Brien Mo.C.323). Of note, many children have viral load higher than adults, helping to explain why some children progress rapidly. (Shearer Th.B.910). Several studies tried to correlate HIV levels in plasma with HIV RNA and infectious cells in the semen and vaginal fluids (Caliendo LB.B.6024, Juriaans Th.A.4036). While higher plasma RNA levels correlated with higher levels of HIV in semen and vaginal fluids, lower levels did not guarantee that the genital fluids were non-infectious. Patients with low plasma RNA levels are still infectious. Other studies tried to correlate maternal RNA levels with the risk of transmitting HIV to offspring. Though higher plasma RNA increased the rate of transmission, there was no critical cut-off below which the risk of transmission was greatly reduced (Brown Tu.B.2374, Burns Tu.C.345). Regardless of viral load, mothers should be offered antiretroviral therapy to interrupt vertical transmission.

Viral Load & Treatment. Virological analysis from several studies demonstrates that patients experiencing a reduction in viral load during the initial weeks of treatment obtain later clinical benefits from this reduction (VA 298, ACTG 116B/117, ACTG 175, CPCRA 007, Delta, Abbott late-stage Ritonavir study, Roche Saquinavir/ddC study). Thus, the ability of viral load changes to predict clinical benefit from a regimen appears well-founded. Yet no studies have yet attempted to maintain viral load below a certain critical threshold, or to use viral load levels to trigger a change in therapy. Such viral load-based strategy trials are now an important research priority. While viral load has emerged as a critical tool in the diagnosis, prognosis and treatment of HIV disease, many patients and providers lack access to these tests or knowledge about how to use them. The province of British Columbia adopted a viral load-based treatment strategy in May 1996, and has secured a price for the Roche Amplicor™ test which is less than half that in the USA ($63 in Canada vs. $150 in the USA). British Columbia recommends treatment for those with CD4 below 500/mm^3 or those with viral load

above 10,000/mm^3, with the goal of reducing viral load by at least 50% (Hogg Th.B.913). The International AIDS Society released recommendations to initiate therapy when viral load exceeds 5,000-10,000 copies and CD4 counts are falling, or when viral load is over 30,000 regardless of CD4 levels. They suggest that treatment should reduce HIV levels by at least 0.5 log, to below 5,000 copies, or to undetectable levels (three very different goals), and recommend use of viral load tests twice at baseline, within 3-4 weeks of changing therapy, and every 3-4 months or in conjunction with CD4 cell tests (Saag et al., *Nature Medicine* 1996 2(6):625-629; updated in Carpenter et al., *JAMA* 1996;276:146-154). These guidelines were based more on guesswork than on research and were immediately outdated by the end of the Vancouver conference, where five studies showed that viral levels could be lowered to undetectable levels for up to 48 weeks using five different regimens in five different populations, ranging from AZT/3TC/Ritonavir or AZT/3TC/Nelfinavir in acutely-infected individuals (Markowitz Th.B.933, L.B.6031), AZT/3TC/Nelfinavir in AZT-naive individuals (Ho Th.B.930), AZT/3TC/Indinavir in AZT-experienced subjects (Gulick Th.B.931), Ritonavir/Saquinavir in AZT-experienced subjects (Cameron Th.B.934) and, perhaps most surprisingly, AZT/ddI/Nevirapine in AZT-naive subjects (Myers Mo.B.294). David Ho demonstrated that, at least in one of these studies, viral load was below 25 copies/mm^3, and possibly at zero, 24 weeks after the initiation of triple-drug therapy. Several other studies indicated that opportunistic infections and immunizations may transiently increase viral load; thus, increasing viral load is not always necessarily a sign of virological failure. For example, bacterial pneumonia raised viral load from 95,000 to 321,000 copies; when the infection was treated, RNA fell to baseline levels (Donovan Mo.B.1379). Gonorrhea increased HIV levels and appeared to accelerate HIV progression (Anzala We.C.3450). Acute tuberculous infection, even when treated, appeared to permanently raise HIV levels, though the African patients in this study were not receiving antiretroviral therapy (Michael We.B.414). Two studies suggested that influenza immunization does not raise HIV RNA levels (Fuller We.B.111, Nelson We.B.113)), while one measured a transient increase, but cautioned that disease resulting from failure to immunize would be far worse for viral load (Ward We.B.114). These studies indicate that opportunistic infection prophylaxis and treatment remain critical to maintaining a low HIV load, and that immunizations should be provided to HIV-infected individuals.

Unanswered Questions. While viral load is an exciting and powerful tool, many questions remain about how best to use it, how often, and what should be the virological goals of antiretroviral treatment. There is a very real danger that people will misinterpret low viral load levels as meaning they are no longer infectious or capable of transmitting HIV sexually, intravenously or vertically. Only 2% of the body's HIV resides in the blood; the rest dwells in the body's lymphoid tissues and in the brain. Most available drugs do not penetrate the blood-brain barrier, so there is a danger that the virus, cleared from the blood and even possibly the lymph nodes, will hide out in the brain until therapy is removed, and then re-emerge to reinfect the immune system. Moreover, CD4 cell increases which accompany viral load reduction do not fill all the holes in the immune repertoire which are caused by HIV-induced immunosuppression. In Vancouver there were many anecdotes of people who stopped prophylaxis or maintenance when their CD4 cells rose ostensibly out of a danger zone; some of whom later developed opportunistic infections. The immune recovery which accompanies profoundly suppressive antiretroviral therapy appears incomplete at best.

VIRAL LOAD ASSAYS

Three Assays

Three viral load assays are available to researchers and to those clinicians whose patients have insurance which covers their cost. New assays are in development, with greater sensitivity, increased ability to detect non-subtype B HIV strains, and other new features.

Three Plasma HIV RNA Assays Compared

Assay/Sponsor	*HIV sequence(s)*	*Linear dynamic range (/ml)*	*Intra-assay consistency*	*Preferred Anticoagulant*
RT-PCR/Roche	*gag*	260- 1,000,000	<0.15-0.33	ACD/EDTA
bDNA/Chiron	*pol*	1,000- 1,600,000	0.12-0.2	EDTA
NASBA/Organon	*gag, pol*	400-40,000,000	0.13-0.23	ACD/EDTA/HEP

ACD = Acid citrate dextran (citrate; yellow-top tube). EDTA = ethylenediaminetetraacetic acid (purple-top tube); HEP = heparin (green-top tube). Taken from Table 1, Saag 1996. Ranges taken from ongoing NIAID Division of AIDS Virology Quality Assurance Program.

Roche Molecular Systems' Amplicor™ HIV-1 Monitor reverse transcriptase polymerase chain reaction (RT-PCR) test is the only viral load test yet approved by the US Food & Drug Administration (FDA). The PCR test magnifies HIV RNA from plasma using reverse transcriptase (RT), the retroviral protein which copies RNA into DNA. [Another protein, called *Taq* polymerase, is used in other PCR tests. Roche is challenging the right of other companies to sell *Taq* (Dickson *Nature* 1996).] The Roche test uses a highly-conserved region from the HIV *gag* protein, and can accurately quantitate HIV-1 subtypes B, C and D, and some subtype A and E strains. PCR requires special laboratory facilities and procedures to prevent contanimation of the RNA signal. After multiple rounds of copying, the resulting RNA signal is labeled and counted. Thus, PCR, like NASBA, uses a signal-amplification technology, in contrast to bDNA, which uses a target-amplification method (see below). According to the FDA, Amplicor™ is 100% sensitive to viral copy counts down to 800/mm^3, and can often detect down to 400 copies. Roche is working on a test, now available only for research, which can detect down to 25 copies/ml. The FDA approved Amplicor™ for diagnosis of HIV infection and for determining prognosis in HIV-infected persons, relying on data from several epidemiological and prospective clinical studies. The FDA declined to approve Amplicor™ for medical monitoring or measuring treatment response because of the lack of sufficient data on how best to use it. Meeting on 21 March 1996, the FDA Blood Products Advisory Committee determined that more studies are needed to optimize the use of viral load for monitoring therapy, and the FDA required such additional studies as a condition of approval.

In Vancouver, Mulder and colleagues from Roche Molecular Systems described successful efforts to

lower the detection limit of the Roche PCR test from 400 copies/ml to 10-20 copies/ml with a dynamic range increased to 3.5 logs. The new method involves centrifuging HIV into pellets at high speed, lysing the pellets in a non-ionic buffer and proceeding directly to amplification. Samples from 55 persons receiving combination antiretroviral therapy, whose RNA levels were lower than 500/ml (the previous limit of detection) were tested using the new, more sensitive assay, and 38% (21/55) were determined to have RNA levels below 10-20/ml (Mulder Th.B.914).

Chiron's Quantiplex™ branched chain (bDNA) test uses a signal amplification technology to detect copies of HIV RNA. The bDNA test uses a suite of highly conserved regions of the *pol* gene taken from 49 divergent HIV isolates around the world. The HIV samples are fixed on a plate, to which light-sensitive probes are added. Each probe can detect a single viral RNA chain. By measuring the readout from the light-sensitive probe, the amount of HIV RNA in the blood can be measured. The bDNA test is based on highly conserved regions of the HIV *pol* gene from 49 isolates taken from around the world. The Chiron test has kept pace with PCR in terms of sensitivity. The first - generation test measured down to 10,000 copies/ml; the current second-generation test (version 2.0) measures down to 500 copies/ml, and the new version 3.0 test can measure down to about 25 copies/ml. Version 3.0 is being shipped to research laboratories around the country and may be commerciallly available in 1997. The bDNA test is simpler than PCR, requiring no special facilities or procedures to prevent contamination during amplification; here the light, not the signal, is amplified. Several large providers such as Bellevue, SFGH and UCSF have chosen bDNA as their preferred RNA test provider. Currently Chiron is negotiating the terms of approval with the FDA; the bDNA test remains available from Corning Clinical Laboratories, SmithKline Beecham Laboratories and Chiron Reference Testing Laboratories. The bDNA test is comparable in cost to PCR.

Organon Teknika's Quantitative Nucleic Acid Sequence Based Amplification (Q-NASBA™) test uses three enzymes -- AMV reverse transcriptase, RNase H and T7 RNA polymerase -- to amplify specific RNA *gag* and *pol* sequences, resulting in an accumulation of RNA particles complementary with the target sequence. After amplification, the product is detected with a labeled probe and measured. Because NASBA uses a smaller sample size (0.1 ml as opposed to 0.2 ml for PCR and bDNA) it is most often used for testing viral load in neonates and young children, as well as in Europe. NASBA appears more sensitive than PCR, and possibly than bDNA, in detecting HIV subtypes other than subtype B (see below). The sensitivity of the current NASBA test is about 400 copies/ml.

RNA in Lymph Nodes vs. Peripheral Blood

Most studies to date have measured HIV RNA in the plasma. However, only about 2% of the body's HIV circulates in the blood; the remainder is in the lymphoid tissues, the mucosa, the spleen, the gut and the brain. HIV levels are 100-1,000-fold higher in the lymphoid tissue (per gram) than in the peripheral blood (Embretson 1993, Faust 1996). While the kinetics of HIV in the plasma are well understood, and the kinetics of HIV provirus are beginning to be determined, much less is known about viral replication and its response to therapy in lymphoid tissues. Several studies in Vancouver explored this issue.

One obvious problem is determining less invasive sampling techniques than surgical biopsy; two options are fine needle aspirate (FNA), in which lymphoid tissue is extracted with a needle, and tonsillar biopsy (Faust 1996). Another practical problem facing researchers quantitating viral burden in lymphoid tissue is determining the denominator. With blood, a cubic milliliter of plasma is the consensus denominator -- what should it be for lymphoid tissue? A given volume, a given number of cells, a given weight, a given germinal center, total DNA, total RNA? These are highly dependent on the mode of sampling (e.g., fine needle aspirate versus surgical biopsy). Consensus protocols for lymphoid tissue sampling will be necessary for the coming generation of so-called 'eradication' trials.

A team from the Vancouver Centre for Excellence in HIV/AIDS and Chiron obtained lymphoid tissues from HIV-infected persons with fine-needle aspirates (FNA) or surgical biopsies. They then used the bDNA test to measure viral levels in the lymph nodes. Among untreated individuals, viral burden in the lymph nodes ranged from 106-109 RNA copies per gram of tissue, a level thrice that seen in plasma (Harris Th.B.915). In treated patients, some lymph node RNA levels went undetectable though the treatment regimens are still blinded (Chernoff 1996).

A Swiss group studied six patients two weeks before baseline, at baseline and one to six months after starting treatment with one to three antiretrovirals. 50% (3/6) subjects had a 2.7-11 fold decrease in plasma RNA at one month and a 7.4-34-fold decrease at six months. Nonetheless, lymph node RNA failed to decline by more than 3.5-fold and never declined as much as plasma RNA. At one month CD4+ T cells rose modestly by 58-129/ml. Lymph node RNA levels did not decrease in two patients with only a transient fall in plasma RNA. The researchers concluded that plasma RNA changes may not reflect changes in lymph node viral burden (Bürgisser Th.B.4329). However, it is likely that the researchers used incompletely suppressive agents, such as nucleoside analogues. It will be interesting to compare peripheral blood to lymphoid changes in viral burden with new, more potent and durable combination antiretroviral regimens.

Around the World with bDNA, NASBA and PCR

The three most-widely used RNA tests differ in their ability to measure various HIV strains from around the world. These differences will become increasingly important in protecting the global blood supply as rarer viruses such as group O become more common, and as other strains found mostly in the developing world, such as A, E, F and G, become more common in North America and Europe, where currently subtype B predominates. More accurate quantitation of variant strains will be important as more people with non-subtype B strains are treated and to study vaccine breakthroughs in studies around the world.

For example, Irwin and colleagues screened 828 patients in Bronx for an HIV 1 and 2 EIA (Genetic Systems) which can detect divergent variants. 40 patients were HIV-infected, and of these, 8 were infected with atypical strains. All, however, screened positive by Western Blot, and so would have been detected if screened as blood donors. Of these, three were infected with subtype A, one with a Thai subtype B strains, two type C subtypes and two had strains non-reactive to all twelve V3-loop peptides screened. Three of these patients were foreign born. All those with residence history had

lived in the Bronx for five or more years. Irwin et al. concluded that "one fifth (8/40) of the newly-identified HIV-infected patients had strains with serologic reactivity that varied from common North American subtype B strains" (Irwin We.C.345).

In Vancouver, Patrick Yeni of Hôpital Bichat-Claude Bernard in Paris, France, described an experiment comparing the sensitivity of bDNA, NASBA and PCR in quantitating nine HIV-1 strains from subtypes A-F. NASBA appeared the most sensitive in quantitating non-B subtypes, while bDNA was moderately sensitive, and the PCR test had difficulty measuring subtypes A, F and G. In France the labeled indication for Amplicor™ PCR states explicitly that it should be used only to quantitate RNA in people infected with subtype B; however, most people aren't subtyped before entering treatment. Yeni commented, "Virologists should know where their patients come from."

A team from Health Canada, Ottawa compared RNA sequences recognized by the Roche Amplicor™ PCR test to sequences from 100 HIV strains from group M subtypes A-G and from group O. There was considerable mismatching with non-subtype B strains, and the authors concluded that "the widespread mismatching found in a number of subtypes severely limits the diversity of HIV-1 detected by the Roche Amplicor system... [which] may cause falsely low readings for patients with viral strains not members of subtype B." (Gleeson Mo.A.160).

An Australian team compared bDNA and PCR for quantitating six HIV-1 group M subtypes, A-F. The tests were blinded, and each sample contained a set amount of HIV antigen. bDNA quantified RNA in all six subtypes. PCR, by contrast, detected subtypes B-F, yet did not detect subtype A, and measured lower amounts of subtypes E and F. Both assays were equally proficient at measuring subtypes C and D. Dunne and colleagues observed that PCR may be limited in its ability to detect non-subtype B strains (Dunne LB.A.6005).

Roche Molecular Systems presented three papers addressing this issue. Christopherson and colleagues examined the effect of mismatches on PCR and found that by lowering the annealing temperature and adding "unconventional bases that stabilize DNA duplexes", mismatched sequences (e.g., those from non-subtype B strains) could more easily be detected. They found that subtype B isolates are efficiently amplified and detected in this system, but that subtypes A and E will be amplified less efficiently (Christopherson Mo.A.161).

Respess and colleagues described a new qualitative PCR test using a smaller blood sample and a reduced annealing temperature. 136 group M and five group O HIV-1 isolates were screened. The modified PCR test was able to detect 100% of subtypes A, B, C, D, F and G, 92.3% (12/13) of subtype E, and 0% (0/0) of group 0 (Respess Th.A.4037). In a similar study intended to optimize PCR for use in Thailand, where subtype E predominates, Young and colleagues screened 566 specimens from 530 babies and 36 mothers. The Roche Amplicor™ PCR test was modified to use a smaller sample and a lower annealing temperature. The test was 100% sensitive, specific and prognostic, detecting all children ultimately determined to be infected, and yielded no false positives. One infant who tested negative on the original test tested positive with the modified Amplicor™ assay (Young Th.A.4035).

It is important to note that the modified Roche PCR assays in development remain qualitative only; that is, they can diagnose HIV infection, but cannot provide quantitative measurements to determine prognosis or monitor treatment. Clearly, further development and standardization of quantitative HIV RNA assays able to detect all strains circulating worldwide are needed.

VIRAL LOAD & PATHOGENESIS

The only way to uncover the kinetic parameters
underlying the steady state is to perturb it.

-- John M. Coffin
"The Population Dynamics of
HIV Drug Resistance," in press 1996

A few years ago, as activists called for intensified basic research on HIV infection (Harrington 1992, Gonsalves 1993), as early intervention with AZT monotherapy appeared to be worse than waiting (Seligmann 1993, Volberding 1995), and as the long-touted promise of combination therapy appeared more elusive than ever (Fischl 1993), several scientific teams made discoveries which helped clarify the pathogenesis of HIV infection, and which led to productive new research approaches.

Originally it had been believed that HIV replicated at only low levels during the period of clinical latency, with higher replication during acute infection and full-blown AIDS (Harper 1986). This view was overturned when three papers appearing in March 1993 demonstrated that HIV circulated in the blood at far higher levels than previously believed, and that even greater amounts of HIV were being produced throughout the period of clinical latency in the lymphoid tissues.

Using one of the first of the new quantitative methods for measuring viral RNA in the blood, the quantitative competitive PCR (QC-PCR) test, a team from GeneLabs and the University of Alabama at Birmingham studied plasma from 66 HIV-infected persons at all disease stages and measured RNA copies ranging from one hundred to twenty-two million per milliliter. They also measured reductions in viral load of up to 235-fold when primary infection resolved or when therapy was initiated. RNA levels were 60,000-fold higher than quantitative microculture levels, suggesting that most virions were defective and replication-incompetent. They also showed that, whereas HIV RNA could be detected in 100% of subjects, p24 antigen, whether immune-complex dissociated or not, and plasma culture were far less sensitive. In general, RNA levels were higher just after acute infection and during CDC-defined AIDS. One patient with 22 million copies during acute infection progressed rapidly to 128 CD4 cells/mm^3 and developed AIDS in spite of therapy. Though AZT-treated patients had initial RNA reductions of around 50%, most returned to near baseline at 7 weeks (Piatak 1993).

Also in March 1993, two research teams published definitive evidence that HIV replicated at high levels in lymphoid tissues throughout HIV infection. Confirming the earlier discovery that HIV was found in lymph nodes (Armstrong 1984, Racz 1986, Tenner-Racz 1986), laboratories from the National Institute of Allergy and Infectious Diseases (NIAID) and from the University of Minnesota showed that HIV replicated at higher rates in lymphoid tissues than in peripheral blood. Using DNA PCR, RNA PCR and *in situ* hybridization, Pantaleo et al. found that individuals who were

asymptomatic had lymphoid viral load 5-10-fold higher than in peripheral blood, while those with symptomatic disease, whose lymph node germinal centers had involuted, had equal amounts of infected cells in both compartments. Viral replication was higher in lymph nodes than blood at all stages of disease. Pantaleo et al. concluded that "a state of true microbiological latency does not exist during the course of HIV infection. The peripheral blood does not accurately reflect the actual state of HIV disease... [which] is active and progressive even when there is little evidence of disease *activity by* readily measured viral parameters in the peripheral blood." (Pantaleo 1993). Soon, viral load assays would provide a readily measured viral parameter in the peripheral blood which would show active viral replication there as well.

In a companion paper, Embretson et al. found that, while 20-34% of germinal center and paracortical lymphocytes had integrated HIV DNA, only 0.25-1.0% of infected lymphocytes had detectable RNA activity. Thus, while viral replication was active, perhaps even more alarming was the much higher number of latently infected cells and the large amount of extracellular HIV trapped on follicular dendritic cell (FDC) networks. No FDCs were found to be infected. Most cells with integrated proviral DNA were CD4+ T cells. In one patient, large numbers of latently infected macrophages were found in the spleen. Embretson et al. conclude that "the extent of infection in the CD4+ lymphocyte population in lymphoid organs where 98% of these cells reside is sufficient to account for a substantial portion of the immune depletion in AIDS by a mechanism of slow elimination of small fractions of cells in which productive infection is continually activated." (Embretson 1993).

In 1994, Fox and colleagues determined that each lymph node germinal center might contain up to 1.2 billion HIV copies (presumably including antibody-bound, extracellular HIV attached to the follicular dendritic cell network) and noted that lymph node viral burden appeared stable over time in serial biopsies, at least until lymph node involution occurred (Fox 1994).

In early 1995 a team from the Aaron Diamond AIDS Research Center, Los Alamos National Laboratory and Abbott Laboratories used the potent protease inhibitor ABT-538 (now Norvir™ brand ritonavir) to exponentially decrease plasma HIV RNA levels. ABT-538 was given at 600-1,200 mg/day to twenty HIV-infected individuals with a median baseline CD4 off 180 (range 36-490) and a median baseline viral load of 134,000 (range 15,000-554,000/ml). Because previous studies had described an apparent "steady-state" between virus production and removal (Meyerhans 1989), it was necessary to perturb this steady state by applying a potent antiviral, in this case ritonavir, which reduced plasma viral burden by over 99%. Thus, the Aaron Diamond researchers could assess how quickly plasma RNA was produced and the rate at which the immune system cleared circulating virus from the plasma. Using the bDNA assay, which at that time had a lower limit of detection of 10,000 copies/ml, they found that ritonavir reduced HIV levels by almost two logs (11- to 275-fold, with a mean 66-fold, or 98.5% inhibition) over thirty days. Ho and colleagues determined that the half-life of viral clearance was 2.1 ± 0.4 days. The researchers estimated that the minimum production and clearance was 50 million to two billion per day in the body as a whole, and that 200 million to 5.4 billion CD4 cells were being created and destroyed each day, with a mean of 1.8 billion cells. Patients with lower initial CD4 counts had more rapid rises in CD4 levels. Ho et al. concluded that intervention should start as early as possible after infection, that antiretroviral activity should be

measured in the initial days, rather than months, of therapy, and that "AIDS is primarily a consequence of continuous, high-level replication of HIV-1, leading to virus- and immune-mediated killing of CD4 lymphocytes." (Ho *Nature* 1995).

Two weeks later, John Coffin, the grand old man of evolutionary retrovirology, published a theoretical paper synthesizing the results of Ho and five other teams which demonstrated concordant results using a number of different therapeutic approaches. Coffin noted that viral load appeared to be at steady-state for months before therapy, and then declined rapidly, with reductions to 90-99% at two weeks after starting therapy. Each study demonstrated a rapid increase in CD4 cells concomitant with the reduction in viral load. Viral RNA rebounded rapidly after drug-resistant HIV strains evolved.

Coffin then proceeded to develop a simple steady-state model of HIV infection, in which virus production, lymphocyte production, virus clearance and lymphocyte death are all in apparent equilibrium for many years, until either resistant virus appears or until immune destruction reaches a certain point, upon which viral levels rise and lymphocyte counts fall inexorably, leading to AIDS and death. In his model, Coffin considered actively infected cells, latently infected cells, chronically infected cells and defective proviruses (he did not consider extracellular, antibody-bound but still possibly infectious virions attached to follicular dendritic cells in lymph node germinal centers). Coffin noted that viral and cell turnover was much greater than hitherto believed, and that between 1,000-3,000 viral generations elapsed in the ten years between infection and disease -- in contrast with viruses such as polio or influenza, which may replicate 10 times within a single host before infecting another person, and with herpesviruses which maintain a true state of intracellular microbiologic latency. Since plasma HIV load declines by up to 99% within two weeks of starting therapy, actively infected cells are clearly producing most of the virus in the blood. If someone has 200 billion CD4+ T cells and about 5% of them are productively infected, than one to ten billion CD4+ T cells die each day. Coffin also observed that viral mutants resistant to any given drug (or combination) may already exist at the start of treatment (Najera 1995), and the addition of the treatment simply amplifies the selective pressure on such resistant mutants to outgrow wild-type, drug-sensitive isolates. Coffin calculated that, on average, every mutation at every codon might occur once per day. However, in the absence of drug, most resistant mutants would be less fit than wild-type. Coffin concluded that, while the rapid appearance of resistance to antiretroviral agents was discouraging, "the results obtained during the course of clinical trials are potentially so revealing that they may well light the way to both a real understanding of the infection process in vivo and to the development of truly effective therapeutic strategies." (Coffin *Science* 1995).

In early 1995, Yunzhen Cao and colleagues from the Aaron Diamond Center demonstrated that long-term non-progressors had low plasma HIV RNA levels and that HIV was difficult to culture from their peripheral blood cells. By then, the lower limit of the bDNA test was about 630/ml. Viral load was below cutoff in 50% of ten long-term non-progressors, and all ten had fewer than one tissue culture infectious dose (TCID)/ml. 80% had less than 100 DNA copies in 100,000 peripheral blood mononuclear cells (PBMCs) and 70% were non-infectious by co-culture. CD8 cells from the long-term non-progressors potently inhibited HIV replication, and their plasma neutralized a broad spectrum of primary HIV isolates. Cao et al. concluded that, "Ideally, therapies should aim to reduce

the burden of HIV-1 to the levels seen in long-term survivors or below, and vaccines should attempt to induce the type of immunity found in these subjects." (Cao *N Engl J Med* 1995).

On tax day in 1995, John Mellors published the first of his papers correlating baseline viral load with prognosis. He studied 62 gay men from the Pittsburgh Multicenter AIDS Cohort Study (MACS) site. Since these results pointed to his later work on 180 and finally on 1,601 individuals, they will be discussed under prognosis (Mellors, *Ann. Intern. Medicine*, 15 April 1995).

On the Ides of March in 1996, the Abbott/Diamond/Los Alamos team premiered the follow-up to its 1995 *Nature* paper. Using a new mathematical model developed after observing viral and CD4 kinetics in five patients given ritonavir (600 mg bid), Perelson et al. estimated that productively infected CD4 cells have an average life-span of 2.2 days (half-life of 1.6 days), and that plasma virions had an estimated life-span of 0.3 days (half-life of 0.24 days). They estimated that 10.3 billion virions are produced daily and that it takes 2.6 days from virion release from an infected cell to productive infection in a second cell. Using their new estimates of viral and host cell kinetics, Perelson et al. predicted that resistance would rapidly emerge to any antiretroviral monotherapy, and that "effective treatment must, instead, force the virus to mutate simultaneously at multiple positions in one viral genome by means of a combination of multiple, potent antiretroviral agents." They concluded that "the 'raging fire' of active HIV-1 replication could be extinguished by potent antiretroviral agents within two to three weeks. However, the dynamics of other viral compartments must also be understood... Each viral compartment could serve as the 'ember' to reignite a high rate of viral replication when the therapeutic regimen is withdrawn." The researchers determined to measure the decay rate of long-lived productively-infected cells and the activation rate of latently-infected cells. (Perelson 1996).

These discoveries, all dependent on highly sensitive viral load assays, paved the way for new, more aggressive antiretroviral treatment strategies which are now bearing fruit. Early in HIV infection, virus production and immune system clearance lead to an apparent, but deceptive "steady-state," in which virus levels and CD4+ T cell levels appear to be in balance. Actually, the immune system is undertaking a heroic, but ultimately unsuccessful, effort to contain viral replication. When Coffin, Ho and Perelson set out, drug therapy appeared capable of reducing the viral load by 50% or more, but only for short periods of time, and was clearly unable to reduce viral levels below limits of detection. This would all change in 1996. In Vancouver, six studies using various combination anti-HIV regimens confirmed Ho and Perelson's data on the kinetics of viral and T cell replication, demonstrating a 90-99% reduction in the first two weeks of therapy, and a slower second-phase decline to undetectable viral levels over the next 12-24 weeks (Ho Th.B.930).

The first phase decay of about two orders of magnitude (two logs, or 99%) represents the loss of plasma virions and circulating productively infected cells. The second phase decay continues down at a slower rate asymptotically towards zero, and represents the loss of latently-infected cells which become activated and release virus, and of chronically-infected monocytes and macrophages. The life-spans of activated T cells, resting T cells, and activated and resting monocytes and macrophages are not well-characterized in the human host, as Simon Wain-Hobson has pointed out:

Very little is known about the dynamics of the human immune system. Certainly the lifespan of memory (CD45RO) and naive (CD45RA) T cells is different. Germinal centers come and go depending on the presence of antigen. T-cell homeostasis would seem possible, but the mechanics and implications for HIV disease are hard to assess. Of course more is known about the mouse, where 30-40% of peripheral immunocompetent T and B cells are renewed every three days (Wain-Hobson 1993).

Using data from the AZT/3TC/nelfinavir study in eleven patients with a median baseline of 245 CD4 cells and 156,000 viral copies, David Ho refined previous estimates from the Perelson model and estimated that the first-phase viral decay half-life was 1.25 days and the second-phase decay half-life was 13.3 days. There was an inverse correlation between CD4 count and lifespan; patients with lower CD4 counts had higher turnover rates and shorter CD4 half-lives (Ho Th.B.930).

The next steps in using viral load to elucidate pathogenesis will include determining the duration for which viral RNA can be held undetectable in the blood, the extent to which plasma RNA reflects lymphoid tissue RNA load, the virological impact of removing therapy in persons who become lymph node negative, and the existence of potential viral sanctuaries such as the brain, gut, spleen or testes. Major technical advances are still required to improve the sensitivity of the RNA tests (ideally down to one RNA particle/mm^3), to standardize and validate various lymph node viral burden assays (e.g., fine needle aspirate vs. surgical biopsy, RNA vs. DNA) and to discover less invasive means of sampling potential viral sanctuary sites.

The Steady State & the Setpoint

In 1996 Perelson et al. developed a mathematical model, based on observation of viral load and T cell kinetics in several studies which reduced circulating HIV levels to 99% of baseline, of the so-called steady state, in which virus production and clearance, host cell production and clearance are all in apparent equilibrium. This equilibrium is illusory, disguising as it does the extraordinarily rapid rate of virus production and clearance, T cell expansion and cell loss. JohnCoffin:

> First, the steady-state virus population decays with a half-time of 1- to 1.5 days. Second, the population of wild type viruses declines to as little as 1% of its initial level after one week. Third, virtually all genomes in circulation after approximately two weeks are mutant, rapidly increasing to near wild type levels. Finally, the CD4 cell number increases with the decline in circulating virus and declines as the mutant virus appears...
>
> The overall average replication cycle is about two days, which corresponds to about 180 generations per year, or 1,000 generations in 5-6 years. Less than 1% of the virus in blood at any time comes from cells infected more than two weeks ago previously, implying, at most, a minor contribution from latently or chronically infected cells...
>
> In order to maintain the steady state there must be a balance between immune response and virus replication, a balance between immune response and antigenic variation, the availability of target cells, or all of the above...

> The amount of virus in blood is thus given by the balance between its transfer from solid tissue and its clearance... At steady state, the number of infected cells is directly related to the rate of infection, which is itself equal to the rate of killing of infected cells.
>
> This concept is very important. *Virus load is not important for its own sake* [emphasis added] -- even the highest estimates of virus production amount to less than a milligram per day. The virus measured in blood is also not centrally important in pathogenesis. Rather, it *is* important as a relative measure of virus replicaion and cell killing -- particularly the number of cells infected and killed by HIV each day. The direct correlation between virus load and progression to AIDS implies that an important factor in progression is cumulative killing of target CD4 cells (Coffin, "HIV Viral Dynamics," in press, 1996).

The steady state is important because the level of circulating plasma HIV RNA robustly predicts the rate of progression to AIDS (see Prognosis, below). Very soon after resolution of acute primary infection, viral load reaches a so-called 'setpoint' which is a reliable predictor of the rate of progression. The steady state is described by the equation:

$$cV = \delta T^* N$$

when

c = the rate constant for virion clearance
V = the virion concentration
δ = the rate constant for the loss of T*
T* = the number of virus-producting cells
N = the number of virions per infected cell (burst size)

In other words, the clearance rate for a given concentration of virus is equal to the clearance rate for infected cell loss times the number of productively infected cells times the burst size. According to Ho, "the values for c and δ do not vary significantly among patients," and so:

$$V \sim NT^*$$

In other words, virus concentration is proportional to the number of productively-infected cells times burst size (number of virions produced per infected cell) (Ho *Science* 1996).

John Coffin writes:

> There are two general mechanisms [maintaining the steady state] that can be imagined: a balance between virus replication and the immune response, and a limitation on the number of available target cells for infection. Arguing in favor of the former hypothesis is the observation that the immune response (particularly the cellular response) seems to play a significant role in the establishment of the steady state following primary infection. Arguing

against it is the stability of the virus load. One would expect a steady state based on the immune response to be inherently unstable, and that at least some of the time, the immune system would prevail and clear the infection, particularly after perturbations such as treatment with antiviral drugs. Furthermore, one would not expect immunostimulatory agents to cause an increase in virus load, as observed.

It is more probable that the viral steady state is maintained by limitation of target cells. Although, at any time, the fraction of CD4 cells that are productively infected with HIV is small, it could easily represent all cells available for infection... This requirement would limit infection to... two types of cells: those specifically stimulated by the immune response and those generated de novo to maintain the correct CD4 cell number. This would have two important effects, both observed. First, the virus load would be sensitive to immune stimulation. Second, as the CD4 cell number declined, the rate of replacement, and thus the number of available target cells would be expected to increase, also leading to an increase in viral load. This provides an explanation for the seemingly paradoxical explanation of increasing levels of virus replication in the face of declining CD4 cell numbers (Coffin, "The Population Dynamics of HIV Drug Resistance," in press, 1996).

In Vancouver, Ho presented additional data demonstrating that Coffin's hypotheses were correct. Ho estimated that, on average, 5% of the body's lymphocytes are activated at any given time, and thus susceptible to productive infection by HIV. As the lymphocyte population drops over the course of HIV disease, the number of activated cells stays about the same and the proportion of activated cells actually increases. Thus, an asymptomatic patient with 33% CD4+ T lymphocytes might have 5% of cells in cycle, a patient with AIDS and 2.6% CD4+ T lymphocytes might have 25% of cells in cycle. Ho measured this by tagging a cell surface marker called Ki67 which is expressed only on cycling cells, and found that the proportion of cycling cells increased as CD4 levels decreased. Activated, replicating Ki67+ CD4+ T lymphocytes might be killed by HIV, killed by the immune response, revert to resting (Ki67-) or continue to proliferate. (Interestingly, CD8+ T lymphocytes appear to cycle at the same rate that CD4 cells do, indicating the existence of a lymphocyte replacement mechanism which is blind or indifferent to the distinction between CD4 and CD8). Therefore, there are just as many targets for infection late in disease, and a greater proportion of cells is susceptible to HIV infection. Coffin explained how this may lead to symptomatic AIDS:

> If all activated CD4 cells are available as targets for HIV infection, than HIV would effectively block their replacement. This effect could be the basis for AIDS. If so, then there would also be a requirement for some other effect leading to loss of pre-existing cells. The CD4 cell decline could reflect simple attrition for natural reasons or another specific effect of the infection process. For example, infection of cells activated in response to an antigen could specifically block replacement of this set of cells, leading eventually to its total loss.

If this were true, three phenomena might be predicted: 1) there would be a preponderance of memory rather than naive T cells after beginning therapy in patients with moderately advanced immunosuppression; 2) the holes in the immunologic repertoire which occur in the course of HIV progression would not be replenished by the increase in CD4+ T cell number after initiating

antiretroviral therapy, and, therefore, people experiencing such increases would not recover their immunity to pathogens for which immunity had been lost before treatment; and 3) therefore the number and proportion of CD4+ T lymphocytes would not return to levels seen in uninfected persons even after long-term suppressive therapy.

The first and third phenomena have already been observed. In Vancouver, David Ho presented data on CD4+ cell phenotypes observed four weeks after initiating therapy in nine patients receiving AZT/3TC/Nelfinavir. Although they registered a modest increase in CD4 counts, most of the new CD4 cells were CD45R0+ memory cells rather than CD45RA+ naive cells [while there is some controversy regarding these markers and the possibility that cells may cycle between being memory and naive, this would not affect their antigen specificity]. Thus, it appears pre-existing pools of CD4 cells are being expanded after the initiation of antiretroviral therapy, rather than new CD4 clones emerging through the thymus. This means that immune reconstitution will be delayed or incomplete. No study so far has documented a return of CD4 cell count or the CD4/CD8 ratio to levels seen before infection. Conversely, however, no study has documented the immunological effects of truly long-term viral suppression, and perhaps, in time, the holes in the immune repertoire will be replenished. Until this becomes apparent, however, the importance of preventing thymic depletion and the irretrievable loss of protective CD4+ cell clones argues for the initiation of antiretroviral therapy before permanent immunologic damage has occurred. At what point this damage becomes irreversible is not yet well-defined. In the meantime, however, clinicians and patients should be careful not to remove necessary prophylaxis and maintenance therapy in spite of CD4 cell increases.

VIRAL LOAD & DIAGNOSIS

Plasma HIV RNA testing has several diagnostic applications. It can be used to confirm a definitive diagnosis of HIV, to diagnose infection during the so-called "window period" before antibodies appear, and for the early diagnosis of infected infants. Most testing centers initially use the ELISA and Western Blot HIV antibody tests, which are cheaper, easier and more accessible than the new viral RNA tests. However, RNA will increasingly be used to supplement antibody and p24 antigen tests used today, as it has the advantage of diagnosing infection earlier.

The "Window Period"

In March 1996, just three months before the FDA licensed the Roche PCR test for screening HIV-infected blood, the CDC released guidelines for the routine use of the p24 antigen test for protecting the blood supply. Currently, 1/450,000-660,000 blood donations per year are infectious for HIV but not detected with available (e.g., ELISA and Western Blot) tests; this comes to about 18-27 donations per year (CDC 1996). In August 1995 the FDA recommended that antibody screening be supplemented with p24 antigen tests, in order to reduce the number of otherwise undetected infectious donations by 25% (or by 7-11 donations; FDA 1995). This is an expensive, and no longer necessary, precaution. p24 testing provides, on average, only six days' advance notice of impending seroconversion, and is far less sensitive than RNA testing. Not all recently infected persons have detectable p24, whereas all have HIV RNA.

In order to protect the safety of the blood supply, blood banks must know the length of the "window period" after infection and before seroconversion. In 1987 the Irwin Memorial Blood Center in San Francisco estimated this window period at 42 days, whereas in 1995, using more sophisticated techniques on a cohort of 31 recent seroconverters, this period was reduced to 18.5-25 days (Petersen 1994, Busch 1995, CDC 1996). In Vancouver, Busch reported that there is a consistent pattern during acute infection, in which first RNA, then p24 antigen and finally HIV-antibodies appear in the blood. RNA tests should be used to diagnose HIV before seroconversion (Busch Tu.A.153).

Early Detection of Infected Neonates

A team from the New York City Perinatal HIV Transmission Study Group collected 109 blood samples from 52 HIV-infected neonates and compared time of detection with RNA and DNA PCR:

RNA vs. DNA for Early Detection of Infected Neonates

Time after birth	*DNA+ (%/N)*	*RNA+ (%/N)*
< 2 weeks	21% (6/28)	39% (11/28)
3-5 weeks	71%	87%
> 5 weeks	87%	98%

RNA was consistently more sensitive than DNA, detecting HIV earlier and missing only one infected infant (DNA missed more). Plasma RNA is the most sensitive current test for early postnatal HIV infection (Brown Tu.B.2374).

VIRAL LOAD & TRANSMISSION

It is unfortunate that, fifteen years into this epidemic, procedures for sampling HIV in semen and vaginal fluids remain in their infancy, and the rate of progress in this field, so important to understanding transmission, is so retarded. Researchers have yet to come to consensus regarding the best available test for measuring HIV in genital secretions, though NASBA seems to be regarded as the front-runner, since it uses a technique to extract highly-charged proteins which inhibit RNA amplification from the sample. Semen includes highly-charged proteins such as spermines which inhibit polymerase activity, potentially reducing RNA load as measured by RT-PCR. Another problem in measuring genital fluid HIV is defining the denominator; with blood, the volume of plasma is used as an easy, agreed-upon denominator (ml or mm^3), but with genital secretions, there is as yet no consensus about whether a given unit fluid volume should be used, or a number of tissue culture infectious doses (TCID) or infectious units per million cells (IUPM). This issue is particularly important because it appears that the strain which is transmitted during sexual transmission represents just a small part of the population of the transmitter's range of quasispecies (Ho 1995, Chernoff 1996).

In Vancouver, several studies addressed the question of whether there was a critical plasma RNA threshold below which a person became non-infectious for sexual or vertical transmission. While several studies documented that higher plasma RNA load correlated with higher RNA and infectious viral load in genital fluids, there was no critical plasma cutoff below which virus became 'undetectable' in genital fluids. Similarly, while higher maternal plasma RNA may increase the risk for vertical transmission, there is no threshold below which transmission does not occur. Therefore, for both sexual and vertical transmission, a low plasma viral load does not indicate lack of infectiousness.

Viral Load & Sexual Transmission

A Dutch group used NASBA to measure HIV RNA levels in semen and blood from 30 HIV-infected persons. In this cohort, plasma RNA levels did not correlate to seminal RNA levels. Moreover, seminal RNA levels themselves did not correlate with the amount of infectious virus detected in semen by quantitative microculture. Neither plasma nor seminal RNA predicts the infectivity of semen. Reducing plasma RNA load does not render someone non-infectious. (Juriaans Th.A.4036).

A team from Massachusetts General Hospital tried to correlate plasma HIV RNA with RNA from cervico-vaginal lavage (CVL) in 22 HIV-infected women. HIV RNA levels ranged from 330-490,000 and could be detected in all the women, whereas only 50% (11/22) CVL specimens had detectable RNA with the NASBA assay (range 820-110,000/ml). 20% (1/5) of women with plasma RNA below 10,000 had positive CVL, compared with 54% between 10,000-100,000 and 75% with over 100,000. Lower CD4 count correlated with higher probability of positive CVL ($p=0.03$). However, there was no statistically significant correlation between higher plasma RNA levels and an increased probability of detecting RNA in CVL. The Boston team did not attempt to correlate infectious cells with RNA in the CVL as the Amsterdam team did with semen. (Caliendo LB.B.6024).

Since Vancouver, anecdotes have circulated about people who believe, on the basis of developing an 'undetectable' plasma viral load, that they are no longer sexually infectious and can safely dispense with the use of barrier methods. These studies demonstrate that this is a dangerous and misleading assumption. While further research on the impact of new regimens on free viral load and infectious cell load in genital secretions is essential, so is behavioral research on the impact of new treatment developments, and public education on the need for continued use of barrier methods.

Viral Load & Perinatal Transmission

A similar controversy has been raging in the field of perinatal transmission -- does higher maternal plasma viral load increase the risk of vertical transmission, and is there a critical threshold below which the risk of vertical transmission is negligible (Burchett 1996, Koup 1996). The answers are yes and no respectively. This only appears contradictory. In addition to high maternal viral load, there are many other determinants of transmission, some of which occur even when maternal viral load is low -- e.g., duration of delivery, bleeding during delivery, etc.

Three papers in Vancouver reported on a correlation between higher maternal viral load and a higher risk of HIV transmission. The Mothers and Infants Cohort Study (MIS) followed 42 HIV-infected pregnant women between 1986-1991 and retrospectively measured their viral load, using the Roche PCR test, during and after pregnancy to assess the association between maternal viral load and risk of vertical transmission. 42 women had paired specimens available to compare viral load before and during the third trimester. 87 women had paired specimens to compare the third trimester and two months post-partum. In neither of these comparisons was there any significant change in HIV copy number over the course of pregnancy and afterwards. Only six of the 138 women were receiving antiretroviral therapy (two transmitted; four did not). The results were significant by the Wilcoxon rank sum test ($p=0.004$) and the logistic regression analysis ($p=0.002$), even when duration of ruptured membranes was accounted for ($p=0.027$) (Burns Tu.C.345).

Association Between Maternal Viral Load & Risk of Vertical Transmission

Maternal RNA/mm³	*N*	*N(%) transmitting*	*Estimated OR (95% C.I.)*
<1,000	20	0 (0%)	0.15 (0.08-2.72)
1,000-9,999	52	7 (13.5%)	1.0
10,000-99,999	48	16 (33.3%)	3.21 (1.21-8.52)
≥100,000	18	6 (33.3%)	3.21 (0.94-11.0)

The New York City Perinatal HIV Transmission Collaborative Study compared viral load in 51 women who transmitted HIV to their offspring and in 54 women who did not transmit. HIV RNA was measured eight weeks before delivery and two weeks afterward. This case-control study used the NASBA test with a lower limit of sensitivity was 2,000 copies/ml. 70% (73/105) women had detectable viral RNA.

Median Viral Load in Transmitting & Non-Transmitting Mothers

Group	*N*	*Mean viral load*	*p-value*
Transmitters (TR)	51	15,300	
Entire cohort	105	10,300	p=0.004 (TR v NTR)
Non-transmitters (NTR)	54	7,200	

22% (7/32) of women with viral load below 2,000 transmitted, compared with 65% (17/26) among the quartile with the highest viral load (>32,000). Stratification for AIDS diagnosis and CD4 count showed that increased viral load was most likely to impact transmission among women who were AIDS-free with CD4 counts over 500, in whom the likelihood of transmission increased 18-fold for every log increase in viral load (Thea Tu.C.344).

Nathan Shaffer and colleagues from the HIV/AIDS Collaboration in Thailand measured maternal viral load at the time of delivery in 342 Thai women and followed their offspring for 15 months to determine HIV status. HIV was subtyped by V3-loop immunoassay and quantified by NASBA. Enrolled mothers had a median age of 22; most (57%) were having their first baby and were asymptomatic (95%). 93% were infected with subtype E. Infection status was determined for 281/295 (95%) infants who remained in the study. Vertical transmission rate was 24.2% overall -- 26% for subtype E and 14% for subtype B (p=0.5). This was not statistically significant, as there were too few subtype B transmissions for a robust comparison.

Risk Factors for Vertical Transmission in 295 Thai Women

Risk factor	*Relative risk for transmission*	*p-value*
Low CD4 count	2.6	0.03
Vaginal delivery	2.4	0.07
Prematurity	2.4	0.02
Viral load >8,000	2.2	0.004

Multivariate analysis revealed that prematurity (RR 4.2, p=0.03) and high viral load (3.0, p=0.007) were independently associated with transmission. The transmission rate for HIV subtype E appears similar (24%) to that of the more intensively studied subtype B. Maternal viral load is just one of many factors which impact on the risk of vertical transmission (Shaffer Tu.C.343).

These studies indicate that, although high maternal viral load increases the likelihood of vertical transmission, viral load below 2,000 copies (in the Thea study) still carries an unacceptably high (22%) risk of transmission. Thus, there is no cutoff for maternal plasma RNA levels below which a woman could be confident that she would deliver an HIV-negative infant. Antiretroviral therapy to prevent vertical transmission should be offered to all HIV-infected pregnant women.

VIRAL LOAD & PROGNOSIS

> Virus load and CD4 counts have very different significance... Imagine the infected but asymptomatic patient as a train rushing along the tracks... unaware that just ahead is a *canyon* with a bridge destroyed by a recent flood. When will they meet their inevitable fate? When will the patient progress to AIDS? There are two variables that need to be known: where the train is now, and how fast it is going. The CD4 count can be imagined as a measure of current distance from progression to AIDS and virus load as a measure of how fast the patient is getting there.
>
> -- John Coffin, "HIV Viral Dynamics," 1996

Viral Load Predicts Outcome

a. Three Studies by John Mellors

In 1995 John Mellors from the Pittsburgh site of the Multicenter AIDS Cohort Study (MACS) published a preliminary examination of the association between baseline viral RNA and clinical outcome using Chiron's Quantiplex™ bDNA test on stored samples from 72 men from the Pittsburgh MACS cohort. In a pilot study, ten men had an unknown date of seroconversion, of whom five progressed to AIDS and five remained asymptomatic after 35-74 months of follow-up (median 59 months). In the pilot study's five non-progressors, all had viral load below 10,000 copies/mm^3 (less than the limit of measurement) and at all subsequent timepoints. RNA levels in progressors were 10,000 in 3/5 subjects at baseline and increased to greater than that amount in all five who progressed to AIDS.

In the second part, 62 men with known dates of seroconversion were studied, including 18 who developed AIDS a median of 3.8 years after seroconversion, and 44 who remained AIDS-free after a median of 5.4 years (maximum 8.3 years). Of these men, 29% (18/62) progressed to AIDS after a median of 3.8 years (maximum 6.5 years). 34% (21/62) had a significant CD4+ T cell drop but did not develop AIDS, and 37% (23/62) had a stable CD4+ count with no symptoms. RNA was compared with serum p24, beta-2-microglobulin and neopterin. RNA proved to be both more sensitive and more potently prognostic than the other surrogate markers. The authors concluded that further increases in the sensitivity of RNA tests would transform them into useful prognostic tools for clinical management of HIV disease. (Mellors *Ann. Intern. Med.* 1995).

In Mellors' second paper, published in *Science* during May 1996, he extended his original investigation to all 180 HIV-infected men from the Pittsburgh MACS site, and found that baseline viral load was highly predictive of clinical outcome and survival at ten years, regardless of baseline CD4 count. Indeed, baseline RNA was the strongest predictor of outcome at ten years. 209 men enrolled at the Pittsburgh site between April 1984 and March 1985. Plasma samples were drawn, processed within

2-20 hours, and stored in heparinized tubes at -70° C every six months. Samples from 86% (180/209) were tested in 1996 using the Chiron bDNA assay with a lower limit of 500/ml and an upper range of 1.6 million copies/ml. Median follow-up ranged from 5.6 years (range 0.02-10.6) in those who developed AIDS to 10.6 years (range 3.2-11.2) in those who remained AIDS-free. Baseline HIV RNA levels ranged from <500 (11 patients, 6.1%) to 294,200 copies/ml. Baseline RNA levels correlated with CD4 levels, but not strongly, so "the CD4+ T cell count in a subject within any CD4+ T cell range was a grossly inaccurate indicator of the level of viremia." Mellors et al. arranged the subjects into quartiles by baseline viral RNA and found a highly significant association between that and progression to AIDS or death (Mantel-Haenszel test, $p<0.001$).

Risk for AIDS and Death by Baseline Viral Load: Mellors 2

Baseline Viral load	*AIDS at 5 years*	*Median survival (y)*	*Proportion Dead @ 5y*
$\leq$4,530/mm^3	8%	>10	5%
4,531-13,020	26%	9.5	10%
13,021-36,270	49%	7.4	25%
>36,270	62%	5.1	49%

Mellors et al. also stratified subjects by baseline CD4+ T cell levels. Only the lowest CD4 quartile had an increased risk of progression or death. The other three quartiles clustered together in the Kaplan-Meier plots. "In contrast to the close relation between baseline viral load and outcome, baseline CD4+ T cell counts failed to show a strong gradient for risk of AIDS or death."

High Initial Viral Load Predicts Death Independent of Baseline CD4 Count: Mellors 2

Baseline CD4 count	*Baseline viral load*	*Median survival*	*p-value*
>500 (median 780)	>10,000	6.8 years	
>500 (median 780)	$\leq$10,000	>10 years	p<0.001
<500 (median 299)	>17,320	5.9 years	
<500 (median 368)	$\leq$17,320	10 years	p<0.001

Mellors et al. compared survival curves in those who had two sequential high titers with those whose second measurement was below the first. They concluded that those with persistently high early viral load had a poorer prognosis. By excluding 29 men whose viral load fell by >80% from baseline by 18-24 months, who were considered to be recovering from acute infection, the proportion estimated with 10-year survival dropped from 20% to $\leq$5% and reduced median survival times from 5.1 to 2.5 years in this quartile. Mellors et al. stated that their study had the longest follow-up of any comparable cohort, that their analysis showed "a marked gradient of the risk of disease progression

and death that was directly related to the initial quantity of virus in plasma," and that now HIV infection can be staged like Hodgkin's disease or colorectal carcinoma. Mellors recommended initiation of antiretroviral therapy in persons with over 500 CD4 cells whose viral load is over 10,000 copies, as 50% of them in his study died within six years.

> Collectively, these data indicate that the extent of viremia, measured as HIV-1 RNA, is the best available surrogate marker of HIV-1 disease progression. Several of the rational criteria for demonstrating the adequacy of a surrogate marker as put forward by DeGruttola et al. appear to have been met: (I) baseline HIV-1 RNA concentrations are highly predictive of prognosis. (ii) There is a strong time-dependent relation between HIV-1 RNA and outcome. And (iii) reduced concentrations of HIV-1 RNA, in response to antiretroviral therapy, are predictive of improved prognosis.

Mellors' paper, while ground-breaking, should not be regarded as gospel. As he mentioned, tubes were processed within 2-20 hours, during which some viral decay might have occurred (e.g., virus may have bound to antibodies), potentially lowering the numbers. Moreover, the use of heparinized tubes lowered the viral copy count by some 30%. Therefore, the Mellors numbers should be regarded as guideposts rather than as absolute triggers, since the distribution by quartile and the medians depended on the unique position of the Pittsburgh MACS cohort at the start of its AIDS epidemic in 1984-85. Moreover, Mellors showed clearly that there is a "gradient," or as he stated in Vancouver, "a monotonically rising curve" of increased risk of progression. The only absolute cutoffs with viral load are assay limits of detection, which have been a moving target throughout the last exciting years of viral load research (Mellors *Science* 1996; Mo.B.533).

Building on his *Science* paper, in Vancouver Mellors presented a similar analysis, masterminded by Alvaro Muñoz of the MACS data center in Baltimore, of the entire nationwide MACS cohort on Wednesday 10 July 1996. The corner conference room was packed to the rafters for the 3:30 p.m. panel on Viral Load at the Vancouver Trade & Convention Center, next to a ocean liner whose elderly, orange life-vested passengers were going through yet another noisy, regimented safety drill. Mellors and colleagues set out to compare the clinical, serologic, cellular and viral correlates of HIV infection in the MACS. Parameters stored at baseline and later measured included HIV RNA (using the version 2.0 Chiron Quantiplex™ bDNA assay with a detection limit of 5-10 copies/ml), CD3+, CD4+ and CD8+ cells, neopterin, beta-2-microglobulin, thrush and fever. All patients who were positive yet AIDS-free at baseline with samples available from MACS visit 3 or 4, 12-18 months post enrollment, in September 1985 on average, and who had follow-up data were analyzed. Of 4,954 men in the MACS, 1,982 were HIV-infected. 83% (1,639) of these were eligible by the definition above. Baseline samples from 1,601 men were available for analysis. Their median age was 33 years. 88% were white.

Baseline RNA Predicts Outcome: Mellors 3

Clinical Outcome	*N*	*Baseline HIV RNA (mm^3)*
Entire cohort	1,601	10,825
Died of AIDS	855	24,200
Developed AIDS	993	19,145
Remained alive	749	4,426
Remained AIDS-free	606	3,636

Mellors and colleagues conducted a multiple regression analysis of the relative hazard for death predicted by RNA, adjusting for neopterin and beta-2-microglobulin; here the median follow-up was about seven years.

Relative Risk (RR) for AIDS & Death by Baseline Viral RNA: Mellors 3

Baseline HIV RNA	*N*	*RR AIDS*	*RR Death*
<500	112	1.0	1.0
500-3,000	229	2.4	2.8
3,000-10,000	347	4.4	5.0
10,000-30,000	357	7.6	9.9
>30,000	386	13.0	18.5

"There is a monotonic, highly significant increase in the relative hazard for AIDS and death with increasing baseline viral load," commented Mellors. The team divided the cohort into quartiles, as they previously did with the Pittsburgh data. Median time-to-AIDS and death were:

Baseline RNA	*Time-to-AIDS*	*Time-to-Death*
Lowest quartile	> 10 years	> 10 years
Second lowest quartile	8.3 years	> 10 years
Second highest quartile	5.5 years	7.5 years
Highest quartile	2.8 years	4.4 years

The MACS-wide data confirmed the Pittsburgh study in that RNA provided powerful prognostic information across all CD4 levels. Mellors, Muñoz and others developed a regression tree stratifying hazard of ten-year progression using RNA, CD4 count, neopterin, beta-2-microglobulin, thrush and fever, in sequence. Mellors suggested that this regression tree would be useful for stratifying patients by risk in order to decide when to intervene. "How early is early?" he asked, echoing many others

throughout the conference. "Intervene with what?" he continued, finally asking, "Do treatment induced changes in viral RNA reduce the risk of progression to amounts observed in this natural history study?" Mellors suggested that they would now, but would not have over the ten years observed by the MACS, when antiretroviral therapy was either non-existent or inadequate. While many of the MACS subjects eventually received AZT monotherapy, Mellors pointed out that its effect on the natural history of HIV infection over a ten-year period was transient and negligible. Mellors reiterated that his numbers should be taken as minimal estimates, commenting, "Our values are depressed by at least 30% compared with values observed today" due to the use of heparinized tubes which may have stood at room temperature for up to 20 hours before being frozen (Mellors We.B.410).

b. Confirmatory Studies

An important question which remained unanswered by the Mellors study was the correlation between plasma RNA, reflecting actively replicating virus, and infectious proviral DNA in circulating peripheral blood mononuclear cells (PBMCs). Would cellular DNA prove as prognostic as plasma RNA in assessing risk of progression over time? Luckily, Neil Graham and colleagues from Johns Hopkins presented a study of DNA as a prognostic marker in 547 Baltimore cohort subjects who were followed for a median of 28 months. Graham and colleagues used p24 quantitative microculture to quantify DNA at baseline and correlate it with clinical outcome in 299 injecting drug users (male and female) from the ALIVE study and 248 gay men from the Baltimore MACS site. 17% of subjects were women, 25% had CD4<200/mm^3, 40% had 200-500 and 35% had >500. Viral DNA was measured in infectious units per million cells (IUPM):

Baseline Proviral DNA Predicts Outcome in Baltimore MACS/ALIVE Cohort

Baseline cellular DNA (IUPM)	*N (%)*	*AIDS-free @ 2 years*	*p-value*
0	17%	92%	
0-16	35%	92%	
16-99	34%	80%	
>100	14%	60%	p=0.0001

The relative risk of AIDS at two years was increased 3.6-fold by a baseline IUPM>100. Infectious viral load predicted survival in those with 200-500 CD4 cells, but not for those with greater than 500. Those whose IUPM were consistently over 100 in the first year of infection fared the worst over time. Graham stressed, as had Mellors, that while an initial viral 'setpoint' predicts progression, there is no precise threshold for increased risk. CD4 counts remained a powerful predictor in this study, because it looked at a shorter period of time than the Mellors study; CD4 remains a powerful short-term predictor of progression and death. It is important to note that the Baltimore team did not look at all proviral DNA, but rather only at that proportion of it (generally a minor fraction) capable of productive infection. Defective, non-infectious provirus was not measured (Graham We.B.411).

In another shorter-term study, researchers with the Swiss HIV Cohort Study collected plasma from 400 HIV-infected persons and followed them from 1991-1993. They were stratified by CD4 count and baseline viral load. Mean follow-up was 31 months. 160 subjects died. 85% survived for one year, and 69% for two years. Mean baseline RNA was 4.34 log and CD4 count 299. Those entering with viral load >4.45 log had an increased relative risk of progression (1.95 RR for those with 4.46-4.92 log, 2.63 for those with 4.93-6.24). Entering with CD4 count < 50 increased the risk of progression (6.53 < 50 cells and 3.33 for those with 50-199 cells). Univariate and multivariate regression analysis was conducted. HIV RNA ranged from 2.0 to 6.2 logs (mean 4.34). All variables were associated with HIV RNA levels. Symptomatic patients, p24 positive individuals, those with a low CD4 count or those with a high beta-2-microglobulin level all had higher viral load than those who were asymptomatic, p24 antigen negative, had higher CD4 levels or lower β2M levels. Plasma RNA and CD4 counts were independent predictors of death (Dubuis Tu.B.2373, Yerly We.B.413).

In a study presented in Vancouver and simultaneously published in *JAMA*, O'Brien and colleagues measured viral load with the Roche PCR test 12-36 months after seroconversion in 188 hemophiliacs with known dates of seroconversion.

Viral Load Predicts Outcome in 188 Hemophiliacs

Baseline viral load	*AIDS-free at 10 years*
<1,000	100%
1,000-9,999	81%
10,000-99,999	48%
≥100,000	30%

Only 8% (15/188) of the cohort were defined as long-term non-progressors (LTNPs) with CD4s still over 500 and no clinical progression. Of these, 5/15 had fewer than 200 RNA copies/ml (TR O'Brien Mo.C.323; *JAMA* 1996).

J.J. Lefrère and colleagues followed 103 French blood donors whose time of HIV exposure was known. 13/103 were lost to follow-up and 25/90 died. Median follow-up was 78 months. Using the NASBA test, Lefrère reported that baseline viral load differences of as little as ½ log had a significant longer-term impact on progression and survival. Having a lower viral load at year one predicted a higher CD4 count at year five (Lefrère Mo.B.1380).

Weverling and colleagues compared 46 individuals from the Amsterdam City Cohort. 23 individuals who died before January 1995 were compared with 23 age- and seroconversion-matched controls. NASBA was used to quantify their viral load from blood drawn six and twelve months post-seroconversion. At six months, viral load was 4.9 in cases and 4.5 log in controls ($p=0.03$); at twelve months it was 4.8 log vs. 4.2 log ($p=0.0005$). CD4 counts and beta-2-microglobulin (β2M) levels were similar in both groups at one year. An early viral setpoint predicts progression, and viral load provides better prognostic information than CD4 or β2M (Weverling Th.B.4330).

One study suggested that CD4 counts remain most predictive for the risk of progression and death over a shorter term. Confirming the prognostic value of low CD4s in the ten-year Mellors study and in the two-year Graham and Yerly studies, a German team followed 130 patients with a baseline CD4 below 100/mm^3 for a median of 41 weeks. Popescu and colleagues compared bDNA and CD4 counts for their ability to predict death. 20.8% (27/130) patients died. Of these, 12.2% had RNA>30,000/ml, 35% had 7,500-30,000 copies and 14% had under 7,500 copies. CD4 counts were more predictive of death in this study than RNA as measured by bDNA in this advanced population followed for about ten months (Popescu Th.B.4298).

Long-Term Non-Progressors Have Low Viral Load

Unsurprisingly, after all this, the results of several studies of adult and pediatric long-term non-progressors (LTNPs) and long-term survivors clearly correlated lower viral load and long-term non-progression and survival. In the San Francisco City Clinic Cohort (SFCC), 623 men have a known time of seroconversion. 21% are estimated to remain AIDS-free 15 years after infection. Non-progressors, without AIDS and with CD4>500 at their latest visit, ten years after infection, make up about 5% of the SFCC; their median period infected is 14 years. These LTNPs were compared with more recent seroconverters, who became infected within the last seven years. LTNPs had more stable CD4 slopes, were less likely to be positive on PBMC co-culture, and were more likely than recent seroconverters to have a viral load below 10,000 copies (Buchbinder Tu.C.553).

In a case control study, a Dutch team compared three groups of ten men whose CD4s were below 200 yet remained clinically AIDS-free (the long-term survivors, LTS), ten moderate and ten rapid progressors. The long-term survivors had a lower median viral load than the moderate and rapid progressors at all time points tested. Year 0 is the year CD4 cells fell below 200:

Viral Load in Long-Term Survivors, Moderate & Rapid Progressors with CD4 Counts Below 200/mm^3 (Log_{10})

	N	*y-2*	*y-1*	*y0*	*y1*	*y2*	*y3*	*y4*	*y5*
Low CD4 LTS	10	4.26	4.02	4.34	4.45	4.36	4.64	4.72	4.68
Moderate progressors	10	4.46	4.75	4.81	4.94	5.66			
Rapid progressors	10	4.51	4.90	4.94					

Small baseline differences in viral load can predict marked differences in the rate of progression in people with severe CD4 depletion (Keet Ireneus Tu.C.552).

Pediatric Viral Load Predicts Outcome

Three studies presented at Vancouver presented that viral load is equally predictive in HIV-infected infants and children as it is in adults. Researchers with the NIH IVIG clinical trial followed 184 HIV-

infected children for a mean 14.3 months and used the NASBA assay to assess viral RNA. HIV-infected children appear to have higher RNA values than adults. The mean baseline RNA count was 566,212 and the median was 270,000. Eight children had more than one million copies/ml at entry. In this 14-month pediatric study, high viral RNA clearly predicted mortality (Mofenson We.B.315).

Pediatric Viral Load Predicts Mortality in 184 HIV-Infected Children

Baseline viral load (NASBA)	*Mortality (median follow-up 14m)*
<4,000	0%
4,001-10,000	2%
10,001-100,000	13%
>100,000	27.3%

A second study followed 150 perinatally-infected children for up to eight years. HIV RNA was measured by NASBA and DNA by PBMC co-culture. 26/150 children were still alive in January 1996 and only 2/26 were severely ill. 7/26 had no or mild symptoms and 6/7 had normal CD4 cell counts. These six children appear to be long-term non-progressors (LTNPs). None were treated before 1991, and only two have received antiretroviral therapy. 8/26 long-term survivors had CD4 counts below 200 and 12/26 had normal CD4 counts. DNA PCR titers were 1 and 11 copies/0.1 ml in two children with normal CD4 counts, and 22 and 260 copies in two children with fewer than 200 CD4 cells. Mean plasma RNA was 490 in 5 children with normal CD4 counts and 2,300 in three children with CD4 below 200. Pediatric long-term survivors, and particularly pediatric long-term non-progressors, appear to have persistently low viral loads (Rakusan We.B.3327).

Shearer and colleagues used PCR to monitor RNA in 106 HIV-infected infants for two years after birth. 13/106 infants died. RNA copy levels ranged from 400/ml, the lower limit of detection, to over 1 million, the higher limit. Copy numbers increased rapidly after birth, peaking at a median of 318,000, and declined slowly to 40,000 at 24 months. Infants from whom HIV RNA was recovered within two days after birth had higher RNA levels than those diagnosed later (780,000 vs. 243,000 copies/ml). Rapidly progressing infants had higher RNA levels than slower progressors. The authors suggested that early therapy may be indicated in infected neonates with high viral loads, and noted that there is no specific threshold predictive of more rapid disease progression (Shearer Th.B.910).

It is unsurprising that HIV infected children have higher viral loads than adults. Their immature immune systems may be less able to contain HIV after acute primary infection, resulting in a higher setpoint and more rapid progression. Moreover, Shearer's data suggests that many infants experience a burst of viremia after birth, either as part of perinatally-acquired acute infection or as a result of immune activation from exposure to other pathogens *ex utero*. However, it is encouraging that some children are able to survive up to 14 years after perinatal infection (CCJ Carpenter, personal communication) and, as the Rakusan data indicate, many more survive up to eight years. The conservatism of pediatric treatment strategies is clear from each of these studies.

VIRAL LOAD & TREATMENT

We fear to raise false hopes.

-- Martin Markowitz
Vancouver, 11 July 1996

John Coffin recently distinguished between three kinds of response to antiretroviral therapy:

> Until about a year ago, all antiviral therapy gave the response in viral load [I label] as "little suppression" in which the rapid decline in virus after treatment is followed by a rapid appearance of mutant virus and more or less complete return to the original set point... More recently, therapeutic regimens which give rise to "partial suppression" have been described... In this case, resistance mutations appear, but they are severely crippled for replication under the conditions of treatment, and they establish a much lower set point, probably with considerable, but not complete, clinical benefit...
>
> Finally, and most recently, apparently complete suppression has been observed with some combination treatments... In this cae, the initial two-log decline in virus load is followed by a more gradual decline to below the limits of detection. (Coffin, "HIV Viral Dynamics," 1996, in press).

Examples of treatment regimens which fit into Cofffin's three patterns are given below:

Antiretroviral Treatment Regimens Conferring Little, Partial or Apparently Complete Plasma HIV RNA Suppression

	Monotherapy	*Combinations*
Little suppression	AZT; ddC; 3TC; NVP; DLV; SQV	
Partial suppression	IDV; NFV; RTV; 1592 (?)	AZT/3TC; AZT/ddI; ddI/d4T; 3TC/d4T (?); AZT/ddC (naive patients)
Apparently complete suppression		AZT/3TC/IDV; AZT/ddI/IDV; AZT/3TC/NFV; AZT/3TC/RTV; AZT/ddI/NVP (naive patients); RTV/SQV

DLV = delavirdine; IDV = indinavir; NFV = nelfinavir; NVP = nevirapine; RTV = ritonavir; SQV = saquinavir.

"Little Suppression" & "Partial Suppression"

There is no need here to go through the disappointing litany of trials demonstrating "little suppression" which have been generated in the nine years since AZT was licensed. Most studies published before 1995 demonstrated "little suppression". Of these, most were of nucleoside monotherapy or nucleoside combination therapy in AZT-experienced patients. In 1995 three studies (ACTG 175, CPCRA 007 and Delta) demonstrated "partial suppression" and demonstrated the efficacy of combination nucleoside therapy, which was more pronounced among AZT-naive patients.

Reduction in Progression with Partial Suppression

	ACTG 175	*Delta*	*CPCRA 007*
AIDS & Death	36%	27%	14%
Death	45%	33%	13%

One recent example will suffice. CPCRA 007 is a good example of a study demonstrating "little suppression", in the case of the AZT-experienced majority of participants (in whom adding ddI or ddC was akin to sequential monotherapy with the added frisson of two-drug toxicity), and "partial suppression" in the minority of AZT-naive participants whose viral load data were presented in Vancouver by Douglas Mayers of Walter Reed.

CPCRA 007 compared AZT monotherapy with AZT/ddI and AZT/ddC in 1,113 patients with fewer than 200 CD4 cells/mm^3 (Saravolatz LD, Mo.B.291). 254 patients were antiretroviral naive and 859 were experienced. Fewer than 4% of patients were lost to follow-up. Median CD4 count and length of prior AZT use were 42/mm3 and 7 months, respectively. Endpoints were progression to AIDS and death, which occurred in 222/366 patients on AZT, 232/372 on AZT/ddC and 242/375 on AZT/ddI. There was no statistically significant difference in time-to-AIDS or death between the three regimens. However, among the AZT-naive stratum, AZT/ddI proved superior to AZT in delaying progression and death (RR=0.54, $p<0.01$). Overall, combination therapy was less well tolerated and no more effective than AZT monotherapy; however, among a subset of naive patients, combination therapy reduced the risk of disease progression and death.

Using the Roche PCR assay, Dr. Mayers examined HIV RNA changes in the AZT-naive subset in 98 patients with blood samples available at baseline. The median baseline CD4 count was 65 and median baseline log HIV RNA was 4.95. At six months, mean decreases in log HIV RNA levels were significantly greater on combination therapy (-0.7 on AZT/ddI. -0.5 on AZT/ddC, -0.0 on AZT). At twelve months the combination arms were still -0.4 log below baseline and the AZT arm was at baseline. The six month difference was statistically significant, and the twelve-month difference was not. In this study, a one log difference in RNA at baseline was strongly correlated with increased risk of progression or death (RR 1.62, $p=0.038$). A one-log decrease in RNA at six months significantly lowered the risk of progression or death (RR=0.39, $p=0.003$) but not for mortality alone (RR=0.61, $p=0.126$). The authors concluded that AZT/ddI confers a greater decline in HIV RNA than AZT

alone, and that baseline HIV RNA and a post-treatment decline in RNA of one log at six months were predictive of clinical response, independent of CD4 counts.

Apparently Complete Suppression of Plasma Viral RNA

At an ICAAC late-breaker in fall 1995, Abbott's Don Norbeck presented a summary of results from a French study of AZT/ddC/ritonavir, in which 24% of patients had undetectable RNA and 30% undetectable infecious PBMCs at four months. At the time, this seemed implausible, and we deemed it an "irresponsible, unsubstantiated claim" (Harrington 1995), but it was the first of a gathering avalanche of reports that documented apparently complete suppression of plasma HIV RNA (Norbeck 1995).

In a late breaker session in Vancouver on July 11, several researchers provided data on new three-drug regimens which, over a 12-48 week period, appeared to reduce viral replication below the lower limit of detection in between 80-90% of participants. These results were seen with several regimens in several populations, including newly-infected acute seroconverters, treatment-naive patients with 50-600 CD4 cells and treatment experienced patients with 50-400 CD4 cells/mm^3:

Studies Reporting That a Majority of Participants Developed Undetectable Plasma HIV Load

Study PI	*N*	*Population*	*Regimen(s)*	*Median follow-up (weeks)*	*Percent Undetectable*
Markowitz	8	Acute infection	AZT/3TC/RTV	40	100%
Markowitz	11	Acute infection	AZT/3TC/NFV	12	100%
Markowitz	9	ARV naive	AZT/3TC/NFV	12	100%
Myers	152	ARV naive	AZT/ddI/NVP	28	70%
			AZT/ddI		40%
Gulick	97	ARV exp.	AZT/3TC/IDV	48	90%
			IDV		40%
			AZT/3TC		0%
Cameron	65	ARV exp.	RTV/SQV	6	50%

[ARV = antiretroviral; IDV = indinavir; NFV = nelfinavir; NVP = nevirapine; RTV = ritonavir; SQV = saquinavir. Limits of detection ranged from <500/ml to <25/ml.]

Over the course of these studies, the limits of detection changed as manufacturers introduced new, more sensitive assays. While initially some studies began with a lower limit of detection of 10,000 copies/ml; newer tests have reduced that level to 100 copies or even, in some cases, 25 copies (Ho). Concomitant with the reduction of viral load to such levels has been a delay in the emergence of virus strains resistant to any of the drugs involved in the regimen (Condra, Th.B.932).

Acute Primary Infection

Martin Markowitz reported on two studies carried out at the Aaron Diamond AIDS Research Center (ADARC) among newly-infected persons with acute primary HIV infection. These studies were designed to study the hypothesis that hitting the virus early, before significant seeding of lymphoid tissues or evolution of potentially drug-resistant mutants had taken place, would increase the likelihood of being able to potentially eradicate viral infection from the body. Each study used a three-drug regimen and reported that, among all patients who could tolerate and remain compliant with the three-drug regimen, viral load became undetectable (below 500 copies/ml as measured by the bDNA version 2.0 test) within twelve weeks, and remained so. In one study, some patients followed out to ten months began to lose their anti-HIV antibody response. The two regimens studied were AZT/3TC/ritonavir and AZT/3TC/nelfinavir.

In the first study, twelve patients enrolled within 90 days of exposure (estimated mean 65 days among those who remembered a potential exposure). Mean CD4 counts were 633 and mean HIV RNA count was 91,359/ml. One patient switched from ritonavir to indinavir after developing an allergic reaction,and three patients were discontinued from the study for non-compliance ('Bad guinea pig!'). Eight remaining patients treated for up to ten months remain plasma RNA negative and are negative by quantitative microculture; infectious virus cannot be cultured from up to ten million peripheral blood mononuclear cells (PBMCs). Eventually lymph node biopsies will be performed to assess whether HIV RNA has been cleared from lymphoid tissues and perhaps one day some of these patients will be offered the chance of terminating therapy to see if they have cleared infection from their body (Markowitz Th.B.933).

A second study, using AZT/3TC/nelfinavir in 12 patients with acute primary infection, demonstrated essentially similar results. Median entry CD4 was 253 and median baseline viral load was 81,000 copies. One patient developed a grade 4 CPK elevation and withdrew from the study; the remaining 11 became undetectable within 12 weeks. While viral load went below 500 copies/ml, median CD4 count rose by 98 at 12 weeks; the median increase was 109 cells (Markowitz LB.B.6031).

Antiretroviral-Naive Patients

Two studies looked at three-drug combinations in middle-stage patients who had not received previous antiretroviral treatment. One study combined AZT/ddI with the non-nucleoside reverse transcriptase inhibitor nevirapine, which previously demonstrated a rapid propensity to induce high-level resistance when used as monotherapy (Richman 1995), while the second combined AZT/3TC with the protease inhibitor nelfinavir.

In the first study, 152 antiretroviral-naive patients with 200-600 CD4 cells were randomized to AZT/ddI or AZT/ddI/nevirapine and followed for twelve months. Baseline CD4 was 376 and viral load was 4.41 logs/ml. At 28 weeks, 70% of patients receiving triple-drug therapy had viral load below the limit of detection (500 copies/ml using the Roche PCR kit), compared with just 40% of

patients on AZT/ddI. Among the patients on triple-drug therapy who did not go undetectable were several who had interrupted their ddI therapy, allowing for the rapid emergence of resistance to nevirapine and AZT (Myers Mo.B.294). The study demonstrates, however, that even three reverse transcriptase inhibitors, which are relatively weak when used as monotherapy, and to which resistance may develop quite rapidly, can provide a potent antiretroviral effect when used together on a rigorously-adhered-to regimen in antiretroviral-naive patients.

In the second study, David D. Ho of Aaron Diamond presented results from a study conducted by Martin Markowitz of twelve antiretroviral-naive patients given AZT/3TC/nelfinavir. Mean baseline CD4 count was 245 (range 26-501) and baseline viral load 56,000/ml (range 14,000-618,000). One patient was lost to follow-up. HIV RNA decreased rapidly by two logs (99%) in the first two weeks of therapy, and descended further towards the lower limit of assay detection of 400 copies/ml (using the bDNA version 2.0 test). At the same time, CD4 counts rose by an average of 100 cells. Ho presented the two-phase viral decay model developed by Perelson et al. (*Science* 1996) and demonstrated that this data, as well as Markowitz's previously-described acute primary infection data, showed curves concordant with the Perelson model. The first-phase decay represents loss of plasma virus and actively-infected circulating cells. The second-phase decay represents the loss of infected macrophages and latently-infected CD4+ T cells. At eight weeks, virus could not be cultured from up to 10 million PBMCs. Using the newer, more sensitive Chiron version 3.0 bDNA assay, which can detect viral load down to a level of 25 copies/ml, Ho demonstrated that at twelve weeks, all eleven patients remaining on study had levels below that threshold, and predicted that they were essentially at zero.

The room became very quiet. You could have heard a pin drop. A collective silent sigh ensued, as the full implications of this sunk in to the thousands of scientists, reporters and activists assembled on this last late-breaker session of the Vancouver conference. People I knew and loved were in this study. Their viral load had been reduced, within three months, to virtually zero. Perhaps some of us would live, after all.

David Ho proceeded relentlessly on, using the prognostic power of the Perelson two-phase decay model to predict that most if not all latently-infected cells could be cleared from the body within a range of 30-120 weeks:

Duration of Treatment Before Viral Burn-Out (in Weeks)

Pool size (Viral copies/body):	10^8	10^9	10^{10}	10^{11}	10^{12}
Decay half-life:					
1 week	27	30	33	37	40
2 weeks	53	60	67	73	80
4 weeks	110	120	133	146	160

Ho stated that there are an estimated one trillion CD4+ T lymphocytes in the body, of which fewer

than 100 billion are activated and thus prone to infection by HIV at any given time. Of these 100 billion, perhaps no more than 10 billion can actively replicate infectious virus. Thus, a reasonable estimate for the "pool size" of HIV produced by the CD4 cells of an infected host is 100 million to 1 billion copies. There are perhaps 1.3 trillion macrophages and monocytes in the body. Fewer than 1% of these are infected with HIV -- something on the order of 1.3 billion cells. 99% of macrophages in the blood turn over (or migrate to tissue) within three weeks. Using these admittedly speculative body-wide estimates, Ho figured that a minimum estimate of the time to viral burn-out would be 30-120 weeks, based on a pool size of one billion copies/day. The estimates were more sensitive to half-life of viral decay than to pool size. Ho also cautioned that if viral replication persisted in sanctuary sites such as the brain), there might be amuch slower third-phase decay, in which case this model's predictions would be over-optimistic. Possible means of forestalling this might include more intensive antiretroviral therapy (four-drug combinations) or activating latently-infected cells with, for example, interleukin-2 (IL-2) (Ho Th.B.930).

Antiretroviral-Experienced Patients

While exciting, the results of the acute infection studies will not impact most currently HIV-infected persons, who have been infected for years. Many of these people are antiretroviral-experienced too, so the exciting results among antiretroviral-naive patients might not necessarily apply to them. Luckily, however, two additional studies presented in Vancouver displayed essentially identical viral load decay slopes among antiretroviral-experienced patients as in the acutely-infected and antiretroviral-naive participants described above. What is most striking about these six studies is that all demonstrate virtually identical viral decay slopes. Thus, they demonstrate the power of the "hit it hard" approach; they do not, however, at least yet, distinguish whether it is indeed better to "hit it early".

Roy "Trip" Gulick of New York University presented 48-week follow-up data from an ongoing Merck-sponsored study comparing AZT/3TC/indinavir to indinavir monotherapy and to AZT/3TC in 97 patients who entered with a median CD4 count of 144 (range 50-400), at least 20,000 copies of HIV RNA (median 41,385), and a mean of 2.5 years of prior AZT therapy. Nine patients developed nephrolithiasis (kidney stones) from the indinavir, and 22 had elevated bilirubin levels.

Proportion of Patients Undetectable in AZT/3TC/Indinavir Study (Th.B.931)

	24w	*32w*	*44w*	*CD4 change at 44 weeks*
AZT/3TC/IDV	92% (22/24)	83% (19/23)	83% (5/6)	+218
IDV	38% (9/24)	36% (8/22)	22% (2/9)	+158
AZT/3TC	0% (0/22)	0% (0/23)	0% (0/8)	+ 14

90 patients remain on study. All have been switched to open-label triple combination therapy (Gulick Th.B.931).

In a second, more preliminary study, Bill Cameron from the University of Ottawa presented six-week data on 65 patients who entered the first two strata of an ongoing study of ritonavir and saquinavir, the first study looking at two-protease regimens. Baseline CD4 counts ranged from 100-500; patients were required to be protease-naive and to discontinue nucleosides before entering the study. The first stratum used a 400 mg dose of each drug twice daily and the second raised the ritonavir dose to 600 mg twice daily. The most common side effects included circumoral paresthesia (numbness around the mouth) in 79% (26/33) of patients, diarrhea, fatigue, nausea and flushing. 50% of the patients who remained on therapy for up to six weeks became undetectable at that time. The study is ongoing, using higher doses (Cameron Th.B.934).

While none of these studies is large enough or has run long enough to demonstrate clinical benefit, taken together they raise the possibility that a treatment strategy of reducing viral load as low as possible may delay progression of disease, restore at least some immune function, and delay the emergence of drug-resistant HIV strains. Longer-term follow-up on larger numbers of patients is needed, and potential viral reservoirs within the body (e.g., lymphoid tissues, brain, gut) need to be assayed to determine whether peripheral blood reductions in viral load are reflected in other body compartments (Coffin 1996; Ho Th.B.930).

PCR vs. NASBA for Assessing Treatment Response

A team of researchers from Amsterdam compared NASBA with PCR in HIV-infected individuals as and after they began antiretroviral treatment. Twenty-four individuals were treated with AZT and there were ten controls. NASBA measured significantly higher baseline values (+0.55 log) than PCR. This apparent difference fell to 0.31 log during treatment. At week four, NASBA measured an 0.94 log reduction in RNA, while PCR measured 0.49. At week 8, RNA was still below baseline on NASBA but not on PCR. Weverling concluded that the two tests should not be used interchangeably, commenting, "In individual patients, the same test should be used over time." (Weverling We.B.412).

Viral Load & Resistance

The global virology elite met in closed session at mountainous Whistler, British Columbia, at the Fifth International Workshop on HIV Drug Resistance from 3-6 July 1996. They were kind enough to deliver a summary of their deliberatons at a roundtable in Vancouver on 9 July. Because this was a roundtable, there are no abstracts to refer to; the Whistler abstracts will be published in a special supplement to *Antiviral Therapy* later in 1996.

In general, the evolution of resistance to antiretroviral agents appears to be closely linked with the three patterns of treatment response identified by John Coffin. The first pattern is a rapid drop in viral RNA followed by the emergence of high-level resistance and the return of mutant viral load to baseline levels. This pattern is seen with nucleoside and non-nucleoside RTI monotherapy. The second pattern is a rapid drop in viral RNA followed by a partial, but not immediately complete, return to baseline. This pattern is seen with therapies which require a stepwise accumulation of

mutations, such as indinavir or ritonavir monotherapy. The third pattern is continued suppression of HIV RNA to such low levels that plasma sampling does not permit the isolation of potential mutant isolates. These studies are perhaps too recent to detect widespread resistance. The hope is that viral replication is being suppressed to such an extent that resistance cannot develop.

Brendan Larder of Glaxo-Wellcome discussed resistance to nucleoside analogues. Foscarnet appears to reverse AZT-resistance. Dual resistance to AZT/3TC may occur, and appears to be linked to a novel mutation at codon 333 of the reverse transcriptase (RT) gene. "A significant proportion of people on prolonged AZT/3TC treatment have dually-resistant virus. There is a strong association between dual resistance and treatment failure." These isolates demonstrate minimal cross-resistance to ddI and ddC, despite theoretical concerns about cross-resistance conferred by the 3TC-induced 184 mutation.

Charles Boucher of Amsterdam discussed transmission of resistant virus, which is increasingly observed. In two Spanish cohorts, 24-30% of seroconverters were infected with AZT-resistant virus (Soriano 1996, Pineda 1996). While the resistant phenotype is slightly less fit than wild-type, it may persist for two to four years in the absence of drug selection, and rapidly recurs at high levels when drug therapy is begun. Boucher stressed the importance of sampling lymphoid tissues as well as peripheral blood. He took a lymph node from a patient whose plasma RNA load was undetectable for 14 months on ritonavir monotherapy, and cultured out virus. The HIV strain was identical to wild-type virus circulating before the initiation of therapy, suggesting (but not proving) that ritonavir suppressed the emergence of a resistant isolate sufficiently fit to replicate. Boucher hypothesized that this wild-type HIV strain persisted in the lymph node as a latent, infectious provirus or as an FDC-trapped virion.

Mark Wainberg presented six-month resistance data from the BI 1046 study comparing AZT/ddI to AZT/ddI/nevirapine. Triple-drug therapy reduced the emergence of resistance to all three drugs. Patients who stopped one of the three rapidly developed resistance to the other two drugs. Wainberg discussed the importance of developing quick, cheap susceptibility assays to determine which of a multiple drug regimen a patient was developing resistance to. Apparently Raymond Schinazi of Emory University is developing a PCR test to genotype HIV DNA and RNA and detect common resistance mutations in RT and protease.

Clyde Loveday presented resistance data from the Delta One substudy, in which 242 AZT-naive patients were randomized to AZT, AZT/ddI or AZT/ddC. 4.4% of these patients had genotypic resistance to AZT at baseline. Interestingly, patients with syncytium-inducing (SI) virus responded better to AZT/ddI than to AZT alone or AZT/ddC.

Phenotypic Resistance at 48 Weeks in Delta One

	AZT	*AZT/ddI*	*AZT/ddC*
Susceptible	15	12	10
Intermediate	7	5	2
Resistant	14	10	19
Total	36	27	19

In Delta One, genotypic resistance to AZT occured more rapidly in the combination arms than on AZT monotherapy. Yet the viral load was much lower on combination, reflecting the greater antiviral potency and hence the higher selection pressure imposed on the virus to mutate. In Delta One, the virologic benefits of combination therapy outweighed the more rapid emergence of resistance.

Doug Richman of UC San Diego presented data on resistance to protease inhibitors. After in vitro passage, all three licensed protease inhibitors have several overlapping resistance mutations:

Saquinavir	L10I	I54V	L63P	A71V	I84V	L90M
Ritonavir		I54V	L63P		I84V	
Indinavir	L10I	I54V	L63P	A71V	I84V	

However, in vivo, saquinavir does not appear to elicit the mutations at 10, 54 and 71 seen in vitro. This may reflect inadequate dosing, absorption and selective pressure. Saquinavir is the strongest protease inhibitor in vitro and the weakest in vivo. It will be interesting to monitor the resistance pattern seen with the new enhanced oral formulation of saquinavir due to be introduced later in 1996, which has ten-fold increased bioavailability compared with the current formulation.

Encouragingly, nelfinavir has a different resistance pattern from those listed above. After in vitro passage, HIV emerges with point mutations at codons 30, 36, 63, 77 and 88. Nelfinavir-resistant isolates remained sensitive to indinavir, ritonavir and saquinavir. In vivo the most common pattern includes 30, 36, 71, 77 and 88 (Patick 1996).

Richman discussed the importance of viral recombination in generating multiply drug-resistant isolates. Recombination, in which sequences from two different HIV genomes are spliced together inside a dually-infected cell, may be a rare event, but it can produce spectacular evolutionary benefits for the virus. Indeed, up to 10% of HIV isolates sequenced worldwide appear to be recombinant. All fully-sequenced E and G subtypes appear to be recombinant. "Recombinant HIV have already established a global reservoir and are responsible for the rapidly expanding epidemic in Southeast Asia" (McCutchan Mo.02). Richman demonstrated the fearsome potential consequences of recombination. Two HIV strains, one highly-resistant to AZT (HXB2) and one resistant to protease inhibitors (LAI), were passaged once in vitro, giving rise to a highly dually-resistant strain. Even if all plasma RNA were suppressed, a recombination event could still occur in vivo if two defective proviral strains inhabiting

the same cell, or in two cells which experienced a fusion event, produced a recombinant virus. This is one means by which the virus may elude efforts at eradication (Coffin 1996 in press). Viral recombination gives HIV the same potential to exploit multiple successful survival strategies which sexual reproduction gives eucaryotic organisms.

Richman also presented an evolutionary tree showing the divergent viral strains detected after autopsy in a person with AIDS. Different body compartments selected for very different viral strains. For example, strains present in the brain were resistant to AZT, but remained susceptible to ddI and ddC, unlike blood strains, which developed resistance to all three. Different strains elicited differing CTL epitopes, indicating that sampling anti-HIV CTL activity in the blood gives and incomplete picture of events elsewhere in the body.

At Whistler, Jon Condra of Merck presented data indicating that HIV strains resistant to indinavir were universally cross-resistant to ritonavir. 60-80% were cross-resistant to saquinavir and the Vertex protease inhibitor VX-478 (Condra 1996). A novel 193L baseline mutation appeared to be correlated with lack of response to indinavir (Eastman 1996).

In Vancouver, Condra presented resistance results from two ongoing studies of indinavir:

Reduced Emergence of Resistance: Merck 019

	AZT	*AZT/IDV*	*IDV*	*p-value*
AZT resistant	64% (11/17)	5% (1/22)	--	p<0.001
IDV resistant	--	18% (4/22)	43% (9/21)	p=0.104 (NS)

Reduced Emergence of Resistance: Merck 020

	AZT/ddI	*AZT/ddI/IDV*	*IDV*	*p-value*
AZT or ddI resistant	63% (10/16)	0% (0/20)	--	p<0.001
IDV resistant	--	10% (2/20)	54% (13/24)	p=0.003

"The combined antiviral effects of indinavir and RT inhibitors dramatically suppressed the emergence of resistance to these agents in a bi-directional fashion relative to the rates observed during [monotherapy]" (Condra Th.B.932).

The I.A.S. / *JAMA* Guidelines: D.O.A.

> The proliferation of competing articulations, the willingness to try anything, the expression of explicit discontent, the recourse to philosophy and to debate over fundamentals, all these are symptoms of a transition from normal to extraordinary research.
>
> -- Thomas S. Kuhn, *The Structure of Scientific Revolutions*, 91

It's always risky to build a house when the ground is shifting under one's feet. Undeterred by the tectonic shifts taking place in HIV pathogenesis and treatment, a self-anointed group of experts from the International AIDS (IAS) Society decided to expand their horizons from simply putting on the biannual AIDS conferences in order to promulate guidelines for the use of viral load-based treatment strategies. Recognizing that their first effort, published in *Nature Medicine* in June (Saag 1996), would be outdated by Vancouver, they released a second effort at the AIDS Conference and published simultaneously in *JAMA* (Carpenter 1996). Demonstrating the difficulty of trying to straddle two horses going through a raging river in opposite directions, these guidelines too were dead on arrival, and should have been subjected to a quick burial after Thursday's late-breaker session where a new treatment paradigm made its spectacular debut. Unfortunately, however, many clinicians and patients will be confused by these guidelines, which are internally inconsistent, vapidly prescriptive and riddled with contradictions. It would be more honest at this point to admit the uncertainties facing research and treatment, and design studies to resolve them.

While the IAS panelists may have been reckless in their over-hasty rush to codify a field in considerable flux, however, they certainly did not deserve to be savaged by a group of psychotic would-be activists from San Francisco, who disrupted an evening panel at Vancouver and dumped fake blood on the panelists. AIDS activism has always rested on a core of non-violence, even in the midst of civil disobedience. People who resort to assault and battery should be prevented from disrupting scientific discussion and debate.

"HIV Viral Load Markers in Clinical Practice": Interim IAS Guidelines, June 1996

	Parameter	*Recommendation*
*	*When to start*	>5,000-10,000 copies/ml & CD4/clinical status suggestive of progression; *or* anyone with >30,000-50,000
*	*Target RNA change after treatment*	Undetectable; *or* <5,000 copies/ml
*	*Minimal decrease showing ARV activity*	>0.5 log decrease
*	*When to switch*	Return to baseline; *or* return to within 0.3-0.5 log of baseline
*	*Suggested frequency of measurement*	2 baseline measurements, 2-4 weeks apart; every 3-4 months *or* with CD4 counts; shorter intervals as decision points are neared; 3-4 weeks after starting/switching therapy

This matrix is rife with omissions and contradictions. What is to be done with people with 10,000-30,000 copies/ml? Nothing is said about this middle group. What is the basis for the cutoff points? Nothing in the Mellors data or anywhere else suggests a critical threshold, rather a monotonically increasing risk. Clearly viral load should be used alongside CD4 count and clinical status. Inventing new thresholds for viral load as was done with CD4<500 and CD4<200 has the potential to create artificial thresholds and create potentially meaningless stratifications. At least CD4<200 had the epidemiological evidence of a clearly increased risk for PCP and other OIs. After Vancouver, there is clearly a difference between 5,000 copies/ml and undetectable levels, so they represent two very different targets for treatment ("partial suppression" versus "apparently complete suppression"). There's nothing wrong with stating the uncertainty and proposing studies to resolve the question. "Partial suppression" may be more acceptable for earlier-stage patients with low viral load and higher CD4s, whereas "apparently complete suppression" may be most desirable for patients at risk for AIDS. The same level of reduction, 0.5 log, is proposed both as a minimal decrease acceptable for showing antiretroviral activity *and* as a threshold indicating treatment failure! ddC rears its ugly head with its half-log viral reduction and its dismal clinical record. An 0.5 log will have a very different outcome for someone with over a million RNA copies than for someone with 1,000 copies/ml. The only people who should be happy with the recommended frequency of RNA testing are the manufacturers of the RNA kits. An orgy of over-sampling is suggested, with no guidance given for other factors -- e.g., intercurrent infection, immunization, immunostimulatory treatment -- which might affect viral load independently of antiretroviral treatment failure and the evolution of drug resistance. No guidance is given for patients whose viral load returns partially, but not completely, towads baseline after having been undetectable. Which drug of a three-drug regimen is failing? How can clinicans and patients find this out without removing each of the drugs in sequence and enhancing the selection pressure for resistance? The authors of the IAS guidelines provide no assistance in resolving these or many other pressing issues, and present the false impression of scientific certainty where none exists.

Let's turn now to the July 1996 IAS guidelines, published July 10 in the *Journal of the American Medical Association.*

Antiviral Therapy for HIV Infection in 1996: IAS Guidelines, July 1996

*	*When to start*	Before irreversible immunologic damage has occurred CD4<500, though some would defer if CD4 350-500 if viral load is <5,000-10,000 copies/ml CD4>500 and RNA>5,000-10,000 (no clinical evidence) Consider for all patients with >5,000-10,000 All symptomatic patients (thrush, OHL, fever, sweats, weight loss)
*	*What to start with*	Most potent regimen for all or reserve for higher-risk patients? Both approaches are defensible. Most potent regimen is two RTIs and one PI, but information on PIs in earlier patients scarce, so most patients should probably begin RTIs

*	*What to start with (continued): first-line antiretroviral therapies*		
		Most defined clinical benefit	*Likely clinical benefit*
		AZT/ddI, AZT/ddC (naive) ddI monotherapy	AZT/3TC, ddI/d4T, 3TC/d4T d4T monotherapy?
*	*When to switch therapy*	Treatment failure: VL rises, CD4 falls, symptoms (?) VL rise to 0.3-0.5 log of baseline; don't measure VL within a month of an OI or immunization Clinical progression Toxicity, intolerance or non-adherence Current use of suboptimal regimen (e.g., AZT monotherapy)	
*	*What to switch to*	Factors to consider: reason for changing, treatment history, current options, disease stage, concomitant medications, cost, availability of reimbursement. *Switching for toxicity/intolerance:* Find a tolerable regimen *For treatment failure:* Find a more potent regimen without cross-resistance *For patients on AZT monotherapy:* add ddI, ddC (?!?), or 3TC; switch to ddI. *For patients on two nucleosides:* add a protease inhibitor and one or two new nucleosides	
*	*When to stop therapy*	In advanced patients when toxicity and quality of life are unbearable and treating opportunistic complications becomes of overriding importance	

In general, the July guidelines more openly admit the confusion and contradictions which face clinicians and people with HIV in 1996, and they frame a series of choices which may provide a useful basis for comparative studies of different treatment strategies. However, after Vancouver, several additional considerations must be mentioned. If someone wishes to go undetectable, they should not start with a regimen likely to induce only partial suppression, such as nucleoside combination therapy. A person who wishes to delay the most potent regimen, avoid the inconvenience of protease inhibitors, or save treatment options for later may do fine for several years with partial suppression. Studies are needed to sort this out. The decisions depend on treatment history, stage of disease, side effects, health care reimbursement and personal choice about how one wants to live one's life. It should be noted that the recommendations are drug or at least class-of-drug specific, whereas comparing different strategies might involve looking at partial versus apparently complete suppression regardless of how one obtained them, as the BI 1046 AZT/ddI/nevirapine study reminds us. As for when to stop, it must be remembered that two recent studies in advanced patients, Abbott's ritonavir + standard of care (SOC) versus SOC study and Roche's study of ddC vs. saquinavir vs. ddC/SQV both showed

clinical benefit in advanced patients. However, in general the IAS guidelines are premature; perhaps they should wait until their next confab in Geneva, by which time things may have settled down.

As the IAS rushed ahead rashly with its immediately-updated, immediately-outdated guidelines, the U.S. NIH and Public Health Service (PHS) postponed indefinitely their plans to convene another State-of-the-Art (SOTA) conference to revise the clearly antiquated 1993 guidelines released after Berlin. The PHS panels have the advantage of bringing outside expertise and objectivity to bear upon the promulgation of treatment guidelines; one problem with the IAS panel is that most of its members have a vested stake in the success of studies they have led, potentially interfering with their ability to temper their renewed enthusiam with an awareness of the compliance, cost, public health and toxicity issues associated with life-long multiple-drug therapy.

Intercurrent Infections Increase Viral Load

Active Tuberculosis Infection Permanently Increases Viral Load. Michael and colleagues from the Walter Reed Army Institute of Research reported on a case-control study carried out in Uganda comparing twenty HIV-infected individuals with active tuberculosis to twenty HIV-positive persons without active TB. At baseline, those with active TB infection had a 5.2-fold greater HIV RNA count using the Roche PCR test (5,335 vs. 1,015 copies/ml). This difference was not significant ($p=0.116$), but they had a 70-fold greater median RNA load at six months (37,285 vs. 539, $p=0.03$), which was significant. All patients had received standard anti-TB treatment, but none received concomitant antiretroviral therapy, which could have changed the outcome. The authors speculate that active TB disease may permanently accelerate the natural history of untreated HIV disease (Michael We.B.414).

Bacterial Pneumonia Transiently Increases Viral Load. Following their report at ICAAC in 1995 that HIV RNA as measured by bDNA increased three-fold with the onset of an AIDS-defining opportunistic infection in 8/8 patients with detectable RNA (Busch 1995), in Vancouver, Donovan and colleagues from the Henry Ford Hospital in Detroit followed viral load using the Roche PCR test in fourteen HIV-infected persons who developed bacterial pneumonia. Viral load was taken an average of 85 days before, then during, and 70 days after the pneumonia occurred. Median viral load before pneumonia was 95,000 copies/ml, during pneumonia 321,000 copies, and afterwards 87,000 copies. 13/14 patients had increased HIV RNA levels during their pneumonia. Neither RNA nor CD4 levels were significantly altered after the pneumonia when compared with before. Clinicians should remember that transient increases in RNA levels may coincide with intercurrent infections, and should attempt to maintain patients on antiretroviral therapy while treating such infections, to control peaks of viral replication (Donovan Mo.B.1379).

Median Plasma HIV RNA Before, During & After Acute OIs

N	*Assay*	*Before OI*	*During OI*	*After OI*	*Ref.*
8	bDNA	18,000/ml	52,000/ml	22,000/ml	Busch 1995
14	PCR	95,000/ml	321,000/ml	87,000/ml	Donovan 1996

Acute Sexually-Transmitted Diseases Increase Viral Load. Anzala and colleagues presented a study of 42 HIV-positive Pumwani sex workers from Nairobi. In this cohort, average progression to AIDS is very short, in the range of four years. Sex workers who used condoms progressed more slowly than others. 42 women were sampled before, during and after sexually-transmitted infections, and their lymphocytes, cytokines and RNA were assayed. Using gonorrhea as an example, the researchers showed that CD4 counts were lower; IL-4, IL-6 and IL-10 levels were higher; and HIV RNA levels were higher, in women with gonorrhea than in those without. Each of these differences was statistically significant. The authors suggest that acute STDs may accelerate the course of HIV infection as well as increase the likelihood of HIV transmission. HIV-infected people should continue having safer sex in order to avoid further exposure to pathogens which may accelerate HIV progression (Anzala We.C.3450).

Do Immunizations Affect Viral Load?

In the past, several teams have reported transient (one-six) week periods of viral activation following immunization (W O'Brien 1995, Staprans 1995). In Vancouver, several papers addressed this topic. Fuller and colleagues from the Boston City Hospital AIDS Program measured HIV RNA levels using the Chiron bDNA assay following immunization with a trivalent influenza A and B subvirion antigen (Wyeth-Ayerst Laboratories). Viral load was assessed at 2, 4, 8 and 12 weeks. The test's limit of detection was 10,000 copies/mm3. Of 68 patients enrolled, 52 had specimens evaluable at baseline and follow-up; 33 subjects had 2-week follow-up data. Only one vaccinee of these 33 had a plasma RNA increase of 0.48 log (three-fold, considered significant with this assay). Among 21 vaccinees the mean RNA change from baseline was 0.014 log at two weeks, and -0.15 log in three controls. While plasma RNA correlated inversely with CD4 count in this study, no interaction between immunization and RNA changes was seen (Fuller We.B.111).

Nelson and colleagues from the University of South Florida measured plasma RNA levels using the Roche RT-PCR test at baseline and 3-5 weeks post-influenza vaccination in 33 HIV-infected adults and compared these to 19 non-immunized controls. The cases and controls were well-matched by age, sex and pre-immunization RNA levels (4.47 and 4.58 $\log_{10}$ in subjects and controls, respectively). Post-immunization RNA levels were 4.62 in subjects; controls maintained a level of 4.56. "There was no significant difference between the pre- and post changes in mean $\log_{10}$ RNA copy numbers between the subjects and controls" (Nelson We.B.113).

Ward and colleagues from Twelve Oaks Hospital in Houston compared CD4 cells, CD8 cells, and HIV RNA levels pre- and post-immunization in 86 HIV-infected patients, of whom 63 were on combination therapy. Patients were immunized with the 1995-1996 Purified Surface Antigen Vaccine, Trivalent (types A and B). CD4 cell levels rose from a mean of 186 before vaccination to 230 and 253 one and two months afterwards, while CD8 levels went from 956 to 1,018 to 914 over th same period. Mean viral load approximated 326,110 at baseline, rose five logs to 979,215 at one month and subsequently fell back to 158,546 at two months. HIV RNA increases were highest in those with over 200 CD4 cells/mm3. Ward suggested that immunization may result in transient increases in viral load, and cautioned that the consequences of an infection which occurs because a

patient was not vaccinated are likely to outweigh the consequences of a transient spike in viral expression coincident with vaccination (Ward We.B.114).

All three papers indicate that the long-term viral consequences of immunization are minimal. Thus, immunization is safer than leaving HIV-infected individuals vulnerable to preventable infections, which can (see above) increase viral load significantly, for extended periods, and possibly (in some cases, e.g., when antiretroviral therapy is not offered, as in the African TB study) accelerate the progression of HIV disease. Therefore, physicians should ensure that immunized patients receive antiretroviral cover to counteract potential temporary viral load increases.

Using Viral Load in Medical Management

In May 1996 the province of British Columbia adopted a treatment strategy of plasma viral load-driven antiretroviral therapy. The Vancouver Centre for Excellence in HIV AIDS recommends quarterly CD4 and viral load tests. When CD4s are below 500/mm3 or RNA over 10,000 copies/ml, antiretroviral therapy is recommended, with a goal of holding viral load below 5,000/ml. The province no longer recommends p24 antigen, beta-2-microglobulin or immunoglobulin tests in this context. Hogg and colleagues from the Vancouver Centre for Excellence in HIV/AIDS studied the cost to British Columbia of viral-load driven treatment. Costs were presented in 1996 Canadian dollars. 1,364 (56%) of 2,444 enrollees were accessed. The previous panel of recommended tests, including CD4 counts, cost $445 (Canadian) per patient year (ppy). 71% of this ($316/ppy) covered CD4 tests. If viral load costs $90 per test, implementing viral load-driven antiretroviral therapy would lead to an incremental cost of $239 per patient year, or $145/ppy if it were done no more than four times a year.

Annual Cost of Viral Load (PCR) in British Columbia

	Cost per test	*Incremental cost per patient year*	*Cost if tested four times yearly*
Canadian dollars	$90	$239	$145
U.S. dollars	$63	$179	$ 97

A Roche researcher was asked how British Columbia negotiated a price less than half the average U.S. price for the Roche PCR test ($150 in U.S. dollars), and replied,"bulk discounts" (Sninsky 1996). U.S. purchasers and third-party payors should negotiate similar bulk discounts for whichever viral load assays they eventually decide to carry.

UNANSWERED QUESTIONS

While viral load is an exciting and powerful tool, uncertainties remain on how best to use it, how often to measure it, and what sort of changes in viral load levels should be sought in using antiretroviral therapy. Undoubtedly further technical developments will improve its ability to helpl elucidate the pathogenesis of HIV disease, protect the blood supply, and monitor participants in treatment and vaccine studies. Among the outstanding issues are:

Assay Development. Further development is needed to increase the sensitivity of commercially-available RNA tests to quantitate plasma HIV to lower limits of detection. In particular, new more powerful assays are needed to quantitate non-subtype B strains, both for research purposes and to monitor viral load in natural history, treatment and vaccine studies. Protocols need to be developed and standardized for the sampling of HIV in lymphoid tissues and in genital secretions.

Pathogenesis. It will be important to determine whether different HIV subtypes are associated with different levels of viral load and differing transmission kinetics. Protocols need to be developed for quantifying HIV levels in the brain, CSF, gut, mucosa, semen and vaginal secretions. The contribution of host factors such as CTLs, soluble CD8-secreted antiviral factors, chemokines, complement, cytokines and neutralizing antibodies to control of viral load over the course of disease remains to be clarified. Persistence of virus in potential sanctuary sites needs to be determined. Further work is necessary to determine the kinetics of CD4 replenishment, the role of thymic and extra-thymic T cell maturation, and whether or not depleted antigen-specific CD4 clones can be repleted after long-term suppressive antiretroviral therapy.

Diagnosis. The US Public Health Service (PHS) should promulgate guidelines for using HIV RNA testing to screen the blood supply for people with acute infection and for detecting non-subtype B HIV strains. Tests should be developed which can detect all prevalent strains circulating worldwide.

Transmission. There is a very real danger that people will misinterpret low viral load levels as meaning they are no longer infectious or capable of transmitting HIV sexually, intravenously or vertically. Further research is necessary to determine the impact of potent antiviral combinations on transmission, to study the transmission of drug-resistant strains, and to develop new prevention strategies and public education messages to avoid an explosion of unprotected sex. Studies of potent combination regimens in pregnant women, neonates and children remain years behind studies in adults, and need to be accelerated.

Prognosis. The natural history of treated HIV infection after 1996 is likely to differ significantly from the natural history of untreated (or inadequately-treated) HIV documented by Mellors from 1985-1995. Natural history and treatment studies should therefore include long-term monitoring of viral load, treatment regimens and ultimate clinical outcomes through both observational and therapeutic research. Prognostic guidelines based on multivariate analysis from completed, ongoing and

prospective cohorts should be developed and regularly updated. As Mellors pointed out, CD4 counts and clinical status still contribute useful information to a multifactorial analysis of the risk of progression.

Treatment. The duration of long-term viral suppression remains to be determined. As Ho has pointed out, there is the possibility of a very slow "third-phase" decay in viral replication, possibly due to antibody-bound, FDC-trapped lymphoid virions or to the persistence of HIV-infected monocytic cells in viral sanctuary sites such as the brain. Potent combination regimens which penetrate the blood-brain barrier need to be developed to avoid the persistence there of isolates no longer found in the blood. The most potent, tolerable regimen(s) for long-term suppression need to be determined, and rational sequences for potent suppression need to be defined. Studies in patients with higher CD4 levels and relatively low viral load need to compare partial versus apparently complete plasma RNA suppression as an initial strategy, or immediate versus deferred suppression. Simpler, more convenient regimens need to be developed to enhance compliance, e.g., with the use of time-release drugs which can be taken once a day or less often. Studies to optimize long-term compliance with complicated, inconvenient regimens need to be conducted, comaring a range of strategies in a diverse set of populations. Interventions which may contribute to replenishment of holes in the immunologic repertoire, and reconstitution of thymic and lymphoid tissue in advanced patients need to be designed and implemented. When and whether people experiencing a significant CD4+ T cell rise after combination therapy can be removed from opportunistic infection (OI) prophylaxis and maintenance needs to be addressed. Finally, research needs to be conducted to determine the feasibility of treating or eradicating HIV disease among infected persons in developing nations.

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