Correlates between CD4 reconstitution in HIV patients on suppressive HAART and soluble markers of inflammation and apoptosis.

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Abstract

Highly active antiretroviral therapy successfully suppresses viral replication and increases CD4+ T cell counts in most patients with HIV infection. However, there is a group of patients which exhibits *discordant* treatment responses, with poor CD4 reconstitution despite successful viral suppression. The clinical consequences of such immunological failure are significant, including both AIDS-related and non-AIDS-related pathology, yet the group is poorly characterised. Little is known about why certain patients do not gain the full benefit of HAART, and there are consequently few therapeutic options currently available to improve their care.

To investigate relevant aspects of the immunopathology of HIV infection in patients on HAART, we have 124 selected patients with persistent viral suppression after 3-9 months of HAART and degrees of CD4 reconstitution ranging from no gains at all to normal CD4 levels. A range of soluble markers of inflammation and apoptosis will be quantified by ELISA technique, in serum samples from each patient from before HAART initiation, and at 6, 12, 24 and 36 months of HAART.

We will seek correlates between rates of CD4 gain and measured levels of cytokines and soluble membrane proteins, during the first 36 months of HAART. Hopefully, the discovery of such relationships may further elucidate the mechanisms underlying discordant treatment responses in HIV patients, and contribute to improved disease management.

Introduction

In most HIV patients, highly active antiretroviral therapy (HAART) successfully counters disease progression by suppressing viral replication and raising CD4+ T cell numbers.[1] Thus, in clinical practice, plasma HIV RNA levels and CD4+ T cell counts are the key parameters in monitoring treatment efficacy. However, some patients exhibit therapy responses in which one parameter improves and the other does not, termed *discordant*. Considering the complexity of the interplay between antiretroviral agents, the virus and the immune system, such variability in treatment outcomes is unsurprising. Nonetheless, the discordant responders clearly warrant further study, as they constitute two as yet poorly characterised groups whose therapeutic needs are not sufficiently met.

An indication of the relative neglect of discordant responders is the fact that there are no universally accepted definitions for virological and immunological success or failure. Consequently, estimates of the frequency of discordant responses vary considerably. A review of the limited literature on the subject described frequencies in the range of 20-30% of patients between 6 and 24 months after starting therapy.[2-4] Definitions of virologic success in included studies ranged from HIV RNA below 50-1000 copies/mL, while definitions of immunologic success included both CD4 gains after a given time and the maintenance of CD4 levels above a given threshold.

The clinical consequences for patients exhibiting discordant responses have been studied to varying degrees. Concerning virologic nonresponders, the evidence is inconclusive,[5] but many studies have established the importance of complete virological suppression for CD4 reconstitution and long term treatment response.[6-8] It is therefore fair to assume that improved virological response would be beneficial this group.

Incomplete CD4 reconstitution, on the other hand, is clearly of negative prognostic significance, associated with an increase in morbidity and mortality due both to AIDS-related events and non-AIDS-related events (these include cardio-vascular disease, liver disease and cancer).[9-15] Increased clinical risk has been shown throughout the suboptimal CD4 range, with a patient's overall prognosis only approaching that of an HIV-negative individual at CD4 levels persistently above 500 cells/mL.[14]

A greater understanding of the immunologic characteristics of discordant responders can likely contribute to improving the care and thus the prognosis of these patients. An opportunity to pursue this goal is afforded us by an extensive HIV patient database at the department of Infectious Medicine at Ullevål University Hospital, Oslo. Having treated HIV patients since 1983, the department has accumulated clinical data for over 2000 HIV-positive individuals, and maintains a bank of frozen serum and plasma samples spanning more than a decade.

Utilising these resources, we intend to conduct a study focusing on the discordant group apparently representing the most significant clinical problem, those with varying degrees of immunologic failure concurrent with good virological responses under HAART. We will investigate possible correlates between immune reconstitution in these patients and soluble factors, quantified in serum by ELISA technique, representing various aspects of immune function and dysfunction in HIV pathogenesis.

Human Immunodeficiency Virus[16]

To contextualise terms and ideas relevant to our study, this section introduces some fundamental aspects of HIV virology and infection.

HIV is in fact two *retroviruses* of the lentivirus class, HIV-1 and HIV-2, sharing a genetic similarity of 40-50%. Both viruses cause AIDS, but HIV-1 is both more pathogenic in the individual and is more easily spread between individuals than HIV-2. HIV-1 is the virus responsible for the global epidemic of the last three decades, whereas HIV-2 is mostly confined to areas of West Africa. HIV-1 is thus the better-studied of the two, and the subject of the study to be conducted.

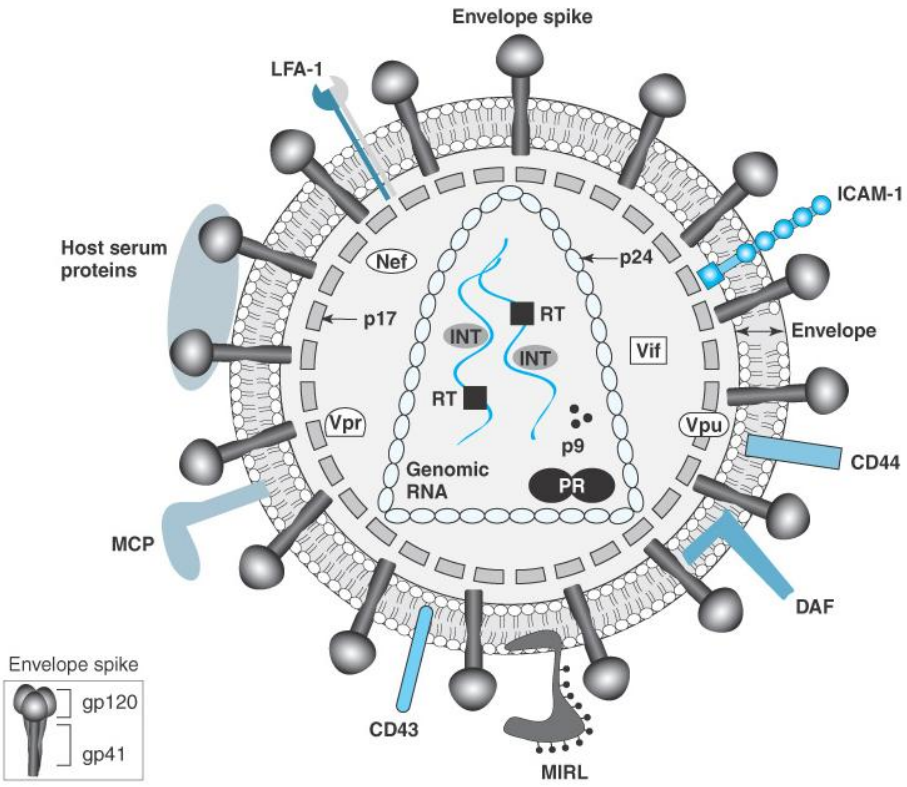
Genome
The genome is a single stranded molecule of RNA, 9.4 kb (9400 base pairs) in length, present in two copies in each virion. When converted to DNA by reverse transcriptase (a hallmark of retroviruses), it encodes nine genes.

Proteins
The nine genes of HIV-1 encode 15 proteins. The three genes *gag*, *pol* and *env*, shared by all retroviruses, each give rise to a polyprotein, which after translation are cleaved by proteases (gag and pol by the virus' own protease, env by a cellular protease) to form functional proteins:

Gag: p17, p24, p9 (structural proteins) and p6 (budding protein)
Pol: reverse transcriptase, integrase and protease (enzymes)
Env: gp120 and gp41 (envelope glycoproteins)

Other HIV proteins are: Tat, Rev, Nef, Vpr, Vpu and Vif (various regulatory functions)

Structure
The virus particle consists of a *core* and an *envelope*. The core consists of the two copies of genome associated with a few molecules of RT and integrase along with the viral protease and the nucleocapsid protein p9, inside a *capsid* consisting of p24, which is itself within a spherical *matrix* consisting of p17. The matrix forms a structural scaffolding for the envelope, a phospholipid bilayer acquired as the newly created virus buds off from the membrane of an infected cell. Anchored in the bilayer is the envelope spike, a structure made up of three copies of a gp120/41 heterodimer.



HIV virion structure **© 2006 Elsevier, Inc**

The main cellular targets of HIV are CD4-expressing T-lymphocytes, dendritic cells (DCs) and macrophages. In addition to the cell surface glycoprotein CD4, a coreceptor is generally required for successful viral entry. Chemokine receptors CCR5 and CXCR4 are considered the most significant (although the use of several other coreceptors has been observed *in vitro*), and various strains of HIV are classified by their use of one or both as coreceptor.

Viral entry

The first step is the binding of HIV gp120 to CD4, which induces a conformational change in gp120, exposing a binding site for the chemokine receptor. (See **(1)**, figure 2) The binding to this coreceptor causes a further change in conformation of gp120, bringing the viral envelope into contact with the cell membrane. The gp41 component of the envelope spike induces fusion of the two phospholipid bilayers, and the viral capsid is released into the cell cytoplasm. **(2)**

Replication

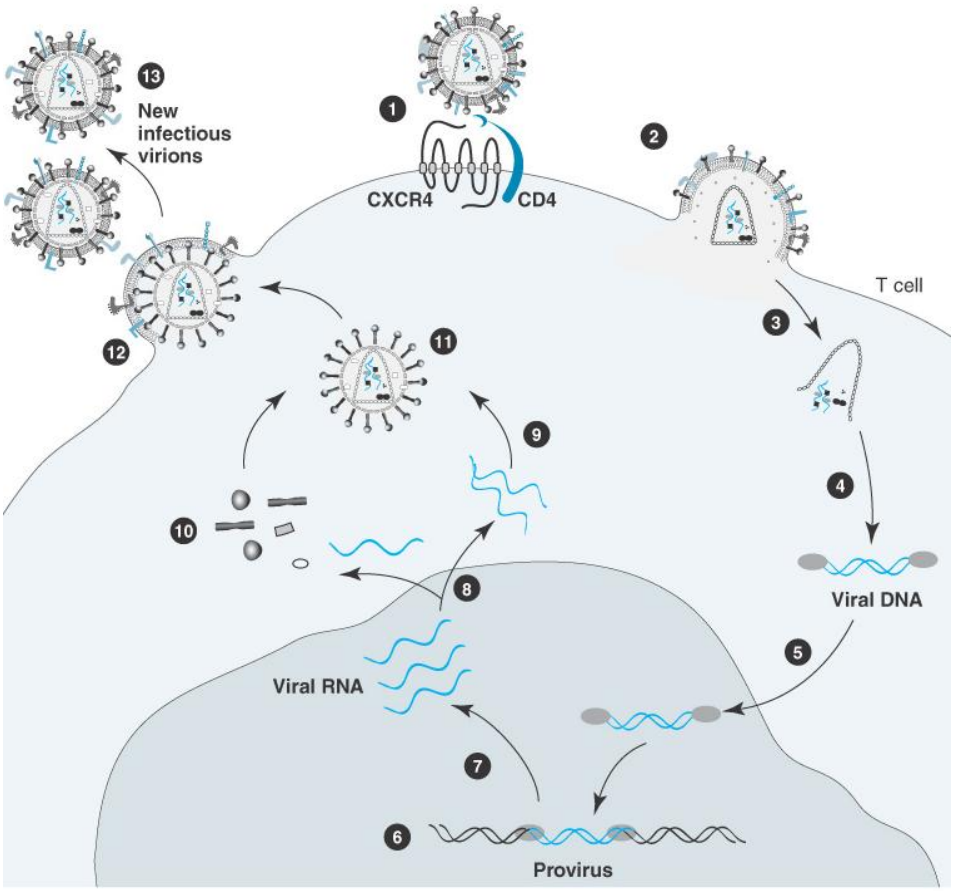
On exposure to the cytoplasm, the capsid is removed by host cell proteases, its contents forming a *preintegration complex,* which heads for the nucleus. **(3)** Inside the complex, the viral RNA genome is reverse-transcribed to complementary DNA. **(4)** The HIV protein Vpr is thought to be particularly important in facilitating the transport of the preintegration complex into the nucleus. **(5)** Once inside, integrase inserts the viral cDNA into a random part of the host cell genome to form the *provirus*. **(6)**

The rate at which the provirus is transcribed depends on whether the infected cell is in a state of activation or resting. In a resting T-cell or unactivated DC, the HIV promoter region is inhibited by cellular regulators, and only a very limited number of viral mRNAs are generated. The number of progeny virions produced in this state of *preactivation* is negligible. However, when the cell is stimulated to activation either by engagement of its T-cell receptor

or by proinflammatory cytokines, it initiates the transcription of many of its own genes, mediated by cell transcription factors which also bind to the HIV promoter. Viral genes are then expressed, including, vitally, the regulatory Tat and Rev. Tat, interacting with several host cell factors, counters the aforementioned inhibition of HIV transcription, increasing the production of viral mRNAs by a factor of several hundred. **(7)** Rev is essential for the export out of the nucleus of mRNAs encoding viral structural proteins and full-length copies of the viral genome. **(8),(9)**

When mRNAs for the structural, enzymatic and accessory proteins have been translated on cellular ribosomes and the resulting polyproteins cleaved by proteases **(10)**, all the elements of new virions are ready to be assembled. P24 forms the capsid around the RNA genome and core proteins **(11)** before reaching the cell membrane, budding through it to acquire its envelope as the progeny virion leaves the cell. **(12)**

HIV is cytopathic through a range of mechanisms, and infected cells in which the virus is actively replicating can only survive for a short time before undergoing apoptosis.



HIV life cycle **© 2006 Elsevier, Inc.**

Clinical course of HIV infection

HIV typically enters the body either through sexual contact or directly via intravenous injection. The first cells to be infected if the virus penetrates the rectal or vaginal mucosa are usually DCs, macrophages and CD4+ T-lymphocytes in the underlying lamina propria. They carry the virus to local lymph nodes, where it finds its way into other resting CD4+ T-cells. Over the following weeks, viral replication increases exponentially, as the infection spreads through the body's lymphoid tissues. 2-4 weeks after infection, a significant number of patients experience a primary illness, exhibiting symptoms that often include fever, lymphadenopathy, fatigue, rash, diarrhoea, headaches and muscular pain.

A vigorous immune response is elicited by the virus, and CD8+ cytotoxic T-cells (CTLs) specific for HIV attack the infected cells. This protective response, along with the cytotoxic effects of the virus, kills an enormous number of CD4+ T-cells during the acute phase of infection, which lasts about 2 months. After this time, viremia subsides, and the chronic phase has begun. Concurrently, circulating HIV-neutralising antibodies are detectable in the patient's serum, and he/she is classified as *seropositive.*

Despite an intense effort on the part of the immune system, HIV is not completely cleared from the body in this early phase, and lies dormant as provirus in macrophages and resting T-cells. A period of chronic infection but clinical latency follows, in which the immune system is under perpetual strain. CD4+ T-cells continue to die en masse despite the relative success of CTLs and antibodies in suppressing viral levels. Hematopoietic tissues churn out replacements at a prodigious rate, but eventually this regenerative capacity becomes exhausted, and CD4-levels fall to the point (usually defined as <200 cells/ml blood) where they no longer provide a sufficient contribution to the defence against microbial pathogens. The opportunistic infections that follow mark the onset of clinical AIDS.

The latency period in HIV infection may last from a few months to over two decades. A host of factors predicting progression to AIDS have been documented, relating to characteristics of both the patient and the virus involved, and patients on each end of the scale have been studied particularly. Those who in the absence of anti-retroviral treatment stay AIDS-free for over 10 years are referred to as *long-term non-progressors* (LTNP), while those who develop clinical disease within two years of infection are called *rapid progressors* (RP).

HAART

Whether disease progression is slow or rapid, HIV infection without treatment is, eventually, almost invariably fatal. While the first HIV drug, zidovudine/azt, was introduced in 1987, it was not until 1996 and the advent of combination anti-retroviral therapy that HIV's assault on the immune system could be combated to any significant extent. By combining several anti-retroviral drugs with different mechanisms of action, viral replication can be reduced in most patients to a point at which the body is able to reconstitute its CD4-levels and thus immune function.

The drugs currently licensed for use can be divided into four groups by mechanism of action:

Nucleoside RT inhibitors (NRTIs): competitively inhibit the deoxynucleosides being strung together to form viral DNA by reverse transcriptase, prematurely terminating the chain.

Non-nucleoside RT inhibitors (NNRTIs): induce conformational changes in RT, inactivating it.

Protease inhibitors (PIs): competitively bind to the active site of viral protease, hindering post-translational cleavage of the gag and pol polyproteins.

Fusion inhibitors: *enfuvirtide*, the only molecule in use to date, binds to gp41, blocking the conformational change which otherwise occurs after gp120 binds to CD4. This prevents the viral envelope from fusing with the cell membrane.

Highly active anti-retroviral therapy (HAART), as it is commonly referred to today, has turned HIV infection into a chronic but relatively stable condition, though only in the countries that can afford to offer it to patients. In addition to a price which is prohibitive in those parts of the world where the treatment is most desperately needed, HAART is demanding of the recipient, requiring near-perfect compliance to effectively suppress viral replication and avoid resistance development. The complicated daily regimens and large numbers of pills involved, along with the significant short and long-term side-effects of the drugs severely test a patient's motivation.
Clearly, the ideal solution, and one which has been pursued intensely since the discovery of the virus, is a vaccine. The goal of bestowing protective immunity against HIV has, however, proved elusive.

Immunopathology

Our study aims to further elucidate aspects of the interplay between HIV and the immune system. This section briefly presents some fundamental elements of the immunopathology of the virus, including some recently established key insights.

T-helper cells (Th cells) are required for the orchestration of an effective immune response, through cytokine production and direct interaction with and activation of CTLs. The preferential infection and killing of this cell population by HIV eventually cripples the defense against microbial infections and many tumours, and is the main marker of disease progression. A phenomenon of key importance, however, is that the loss of Th cells extends far beyond those actually infected by virus. Several mechanisms have been suggested to explain this loss, including upregulation of several pro-apoptotic membrane proteins on infected cells and interference with T cell maturation. An observation which has proved

highly significant is that the majority of Th cells lost through all phases of HIV infection are from the gastrointestinal tract. This has been linked to the persistent immune activation of chronic HIV infection.

HIV-specific CTLs kill infected CD4+ cells by perforin/granzyme-mediated cytolysis and release soluble factors which inhibit viral replication, such as *RANTES* (CCL5), along with a range of other cytokines. In the acute phase of infection, the CTL response is vigorous, but as the disease progresses and Th function is lost, CTL response suffers accordingly. An additional pressure on the CTL population is the frequent generation throughout the disease course of so-called *genetic escape mutants*, HIV viruses displaying new epitopes. This is a result of the high error rate of viral reverse transcriptase along with the frequently high rate of replication of HIV. Consequently, ever new CTL clones are activated, over time causing *clonal exhaustion*.

A new concept in relation to T-cell responses is that of polyfunctionality and response quality. It was discovered that HIV non-progressors had T cells which exhibited the ability to perform many functions at once (i.e. release many different cytokines and perform cytolysis), compared to progressors. This response *quality* has correlated better with viral suppression than T cell *quantity*. The induction of such polyfunctional T cell responses has become a goal in vaccine development.

Another consequence of the high mutation rate of HIV is the relative inefficacy of the humoral immune response, that mediated by antibodies. The virus is present in so many antigenic varieties that a neutralising antibody response becomes impossible. In addition, it is thought that key epitopes in the proteins of the viral envelope are sterically obscured, hindering those antibodies that would bind the virions between cells from doing so. Finally, multiple clones of B cells are activated by gp120 acting as superantigen, producing antibodies which have no effect against the virus. As with CTLs, this is thought to cause clonal exhaustion, constituting yet another immune defect in advanced disease.

Chronic immune activation is a fundamental feature of progressive HIV infection, and has numerous deleterious consequences. Already mentioned is the polyclonal activation of both B and T cells, putting strain on their homeostatic mechanisms. Over time elevated levels of proinflammatory cytokines damage lymphoid tissues, including the thymus, site of T cell maturation. Most detrimental is probably the fact that the large numbers of T cells being activated throughout the disease are prime targets for the virus to infect.

The reason for this immune activation has recently been linked to the clinically apparent enteropathy that has long been recognised in HIV infection. The GI tract normally houses the majority of the body's lymphocytes, but it has been found that during acute HIV infection, most of the CD4+ cells there are killed directly by viral infection. This local depletion continues throughout the disease course. At the same time, there is increased apoptosis of enterocytes and increased intestinal permeability. Recently, increased levels of *lipopolysaccharide* have been found in the blood of HIV-infected individuals, indicating a translocation of microbial products across the damaged intestinal mucosa. While the immunostimulatory effect of LPS is mediated first and foremost via *Toll-like receptor 4*, it is assumed that other microbial products similarly enter the blood-stream, activating the immune system through other means.

Methods

Subject of investigation
Our study question can be summarised as follows: Is there a correlation between CD4 reconstitution in patients on HAART with undetectable HIV-1 viral loads and soluble markers of inflammation and apoptosis?

Patients
We have selected patients from the HIV database at the Department of Infectious Medicine at Ullevål Hospital who meet the following inclusion criteria:

- initiated HAART after 01.01.2000
- achieved viral suppression to undetectable levels (<50 copies/mL) within 3-9 months of HAART initiation
- maintained viral loads under the limit of detection throughout the study period, allowing for single blips.
- no AIDS-defining events before or during the study period

Time period
Serum samples have been selected, where available, at baseline (last available sample before initiation of HAART) and at 6, 12, 24 and 36 months after HAART initiation. No samples dating after November 2007 are available, as serum/plasma storage for research purposes at the department ended at that time. The number of samples available for each patient varies from 2 to all 5 points in time.

Soluble factors
At the time of writing, the final decision about which soluble factors are to be investigated is yet to be made. Several issues influence this choice.

Firstly, we are fortunate enough to be working with a laboratory which performs ELISA analyses with a high degree of automation, handling large volumes of samples simultaneously. This reduces analysis time and cost, enabling us to choose several soluble factors to analyse.

On the other hand, the number and variety of analyses is limited by the amount of sample material available, 2 vials of frozen serum for each patient from any given time point.

Additionally, we are limited in our choice by the time and likely suboptimal temperature at which the samples have been frozen. For the results of the ELISA analysis to be reliable, we must choose soluble markers known to be robust when frozen over long time periods.

Many molecules are of interest:[17]

RANTES (Regulated upon Activation Normal T-cell Expressed, and presumably Secreted): also known as CCL5, this chemokine is secreted by activated T lymphocytes and is chemotactic for other T cells, eosinophils and basophils. Along with MIP-1-α and -β, it is a ligand for the CCR5 chemokine receptor, and thus functions as an HIV inhibitory factor by preventing viral entry into cells.

MIP-1-α and **MIP-1-β**: also known as CCL3 and 4, respectively, these two chemokines are produced by macrophages when stimulated by bacterial lipopolysaccharide. They contribute

to local inflammatory responses by activating granulocytes, and together with RANTES inhibit HIV entry into cells.

TNF (Tumor Necrosis Factor): Secreted by monocytes, macrophages, neutrophils, NK cells and certain T cells upon stimulation by LPS and a range of cytokines, including interferons and interleukin 2. This key inflammatory cytokine has specific receptors on nearly all cells in the body, and a wide range of biological effects. Chronically raised levels of TNF play a role in the chronic immune activation of HIV infection. Also of interest would be measuring circulating levels of soluble TNF receptor, or **TNF-BF (TNF-Blocking Factor)**, believed to regulate TNF effects by preventing the cytokine's interaction with cell receptors.

TRAIL (TNF-Related Apoptosis-Inducing Ligand): Part of the TNF ligand superfamily, this cytokine can induce apoptosis by binding to Death Receptors 4 and 5. There is evidence to suggest that HIV-induced expression of TRAIL by antigen-presenting cells contributes to the extensive apoptosis of uninfected CD4+ T cells.[18]

sFas/sFasL: Fas is an important component of the extrinsic pathway of programmed cell death. HIV-infected cells upregulate their expression of Fas ligand, inducing apoptosis of their uninfected neighbours.[16] Soluble Fas and Fas ligand are often elevated in HIV infection, possibly evidence of Fas-mediated depletion of CD4+ T cells.[19]

IL (interleukin)-17: Contributing to enterocyte homeostasis, this cytokine is expressed by a population of Th cells (Th17) which is preferentially lost from the GI mucosa in HIV infection.[20] This may contribute to the increased permeability to microbial products such as LPS which is implicated in chronic immune activation and HIV disease progression.

IL-18: Expressed in a variety of immune cells, this proinflammatory cytokine induces the expression of IFN-γ, which in turn promotes so-called Th1 responses as well as upregulating Fas-expression.

IL-7: Synthesised by stromal cells in the bone marrow and thymus, IL-7 has been identified as an important growth factor for T cells. IL-7 levels tend to be elevated in HIV patients and inversely correlated with CD4 counts, [21] indicating the existence of a direct feedback loop that may be critical to CD4 reconstitution under HAART.

ELISA

The soluble factors under investigation will be quantified in the serum samples by ELISA, at the laboratory of the Research Institute for Internal Medicine at Rikshospitalet, Oslo.

ELISA, or enzyme-linked immunosorbent assay, is a powerful and widely used method to detect and measure concentrations of specific proteins in complex mixtures. Developed in the 1970s as an immunological tool, it has also found use in medical diagnostics, toxicology and quality-control in various industries.

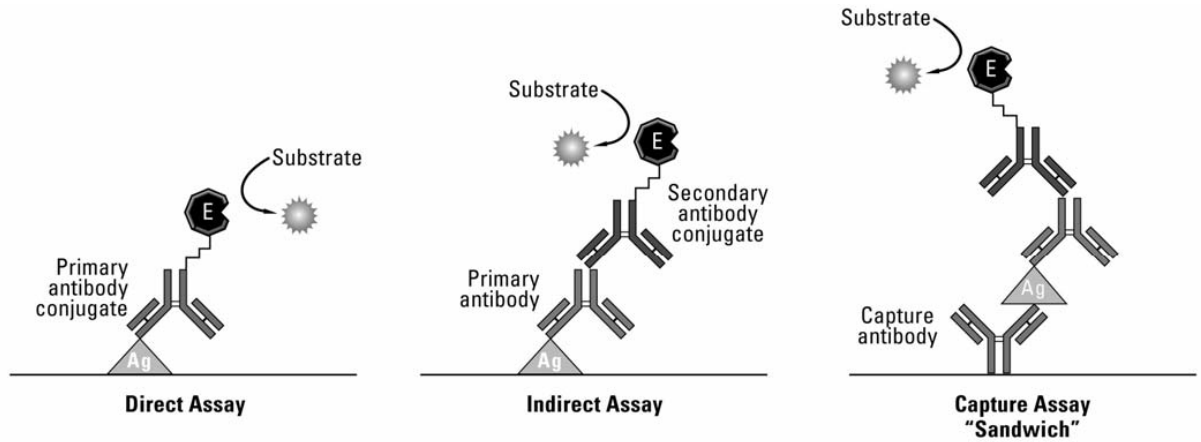
While many variations on the method exist, the fundamental elements are: [22]

1. Coating/capture: The antigen is immobilised in a well of a *microtiter plate*, either directly, that is, by passive adsorption (in a traditional ELISA), or by the use of a capture antibody (in "sandwich" ELISA). The capture antibody is specific for the antigen of interest, and promotes the preferential binding of this antigen in the well. This is useful where the antigen is in a complex solution with many other proteins(such as serum/plasma?)

2. Plate blocking: To prevent non-specific binding of the detection antibody and/or secondary antibody (if used) to the well, all vacant binding sites are covered by an irrelevant protein or other molecule, often bovine albumin.

3. Probing/detection: An antigen-specific detection antibody is added to the wells, forming a high-affinity bond to the antigen. In direct ELISA, the detection antibody is tagged with an enzyme which catalyses the colour change to be measured. In indirect ELISA, by contrast, the enzyme is attached to a secondary antibody which binds the Fc-fragment of the detection antibody. This enables an amplification of the signal, improving the sensitivity of the assay. Then a solution of a chromogen substrate for the enzyme is added, and a coloured product is generated, in an amount proportional to the concentration of antigen present in the sample.

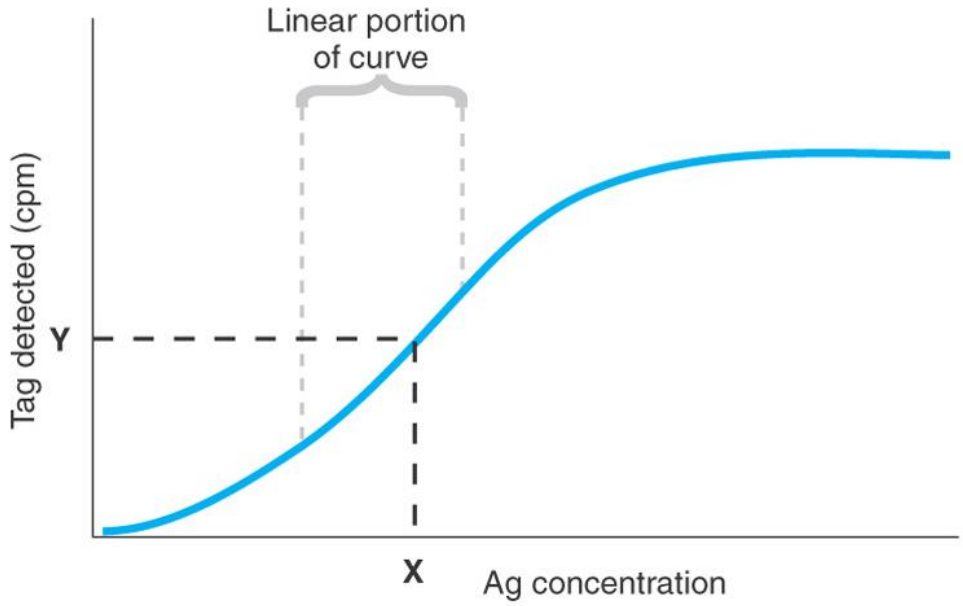
4. Signal measurement: The intensity of the colour change is measured by a spectrophotometric plate reader, and the concentration of antigen extrapolated from a standard curve for the assay.



Some typical ELISA configurations **© 2010 Thermo Fisher Scientific**

In our study, samples will be analysed by sandwich ELISA, using Horse Radish Peroxidase (HRP) as the enzyme tag. For each assay, antibodies and antigens standards need to be purchased. The quantitation of certain soluble markers, such as IL-7, may require the use of specific, high-sensitivity kits, with special signal amplification.

For each assay, a standard curve will be generated by analysing serial dilutions of a known concentration of reconbinant antigen, to which the sample signals may be compared and antigen concentations extrapolated.



Example of an ELISA standard curve

Statistical analysis

The possible correlation between ΔCD4 (CD4 reconstitution from baseline) and the quantities of soluble factors at each time point will be investigated using parametric statistical methods. The main prerequisite for using parametric methods is that we can assume that the sample is normally distributed, which depends upon the types of variables in our data set and *n*, the size of our sample.

If the necessary assumptions about normal distribution can be made, parametric statistical methods are preferable to non-parametric methods because they are more sensitive in uncovering statistical relations between the variables.

Progress

149 patients conformed to the selection criteria, but 25 had to be eliminated from the study due to missing samples. Thus, at the time of writing, a total of 520 serum samples from 124 patients have been selected, and are to be sent to Rikshospitalet for analysis as soon as the final decision has been made regarding the markers to be investigated.

Remaining work

A sample size of 124 patients is most likely large enough to allow the use of parametric statistical methods to explore the data from the ELISA (marker concentrations) analyses and the HIV database (CD4 counts and time periods).

We will seek statistically significant differences in levels of soluble factors between patients exhibiting different levels of immunological response to HAART. Combined with existing knowledge about the roles of these signalling molecules and receptors in normal immune function and HIV pathogenesis, such correlates may hopefully contribute to improved care for immunologically discordant patients in the future.

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